ANALYTICAL STUDIES OF ORGANIC COMPOUNDS OF PHARMACEUTICAL IMPORTANCE

SUMMARY

OF THE

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

Doctor of Philosophy

IN

APPLIED CHEMISTRY

BY

SAMEEN LAEEQ

Under the Supervision of

PROF. ALI MOHAMMAD

DEPARTMENT OF APPLIED CHEMISTRY
FACULTY OF ENGINEERING & TECHNOLOGY
ALIGARH MUSLIM UNIVERSITY
ALIGARH (INDIA)

2010

SUMMARY

The work summarized in this thesis involves the use of thin layer chromatography (TLC) as an analytical tool for identification and separation of organic compounds of pharmaceutical importance such as amino acids, vitamins, carbohydrates and nucleobases. In quest of an inexpensive methodology for the identification and separation of organic compounds, several chromatographic system involving novel eluent have been developed. The results presented in the thesis contribute substantially to the advancement of normal-phase TLC procedures. The interesting features of the present study are:

- Use of commercially available silica gel and cellulose sorbents as layer material along with precoated high performance thin layer chromatography (HPTLC) plates.
- Use of mixed aqueous micellar surfactant solution and microemulsion as the component of mobile phases to investigate its role in modifying the retention pattern of analytes on TLC plates.

The present work formulated with the objective of developing new TLC systems comprising of novel mobile phases for achieving improved separations of organic compounds of pharmaceutical importance. The results obtained during present study have been encapsulated in the form of six chapters of the thesis.

Chapter-1 An introductory part summarizes brief history of chromatography, a comprehensive description on the use of thin layer chromatography as an analytical technique, usefulness of surfactants as an eluent in chromatography. Besides, it also provides a general idea about the amino acids, vitamins, carbohydrates and nucleobases, along with their classification and structural formulae. Moreover, a detailed description of TLC alongwith complete literature survey (1995-2009) on application of chromatographic techniques to amino acids, vitamins, carbohydrates and nucleobases has been provided in this chapter.

Chapter-2 Thin layer chromatography of eight essential amino acids has been performed on silica layers with seventeen mobile phases of different compositions. The mixed aqueous surfactants solutions Triton X-100, 1.0 x 10^{-5} M-sodium dodecyl sulphate, 8.1 x 10^{-4} M - acetone 1:1:5 (v/v/v) was identified as the best mobile phase for specific separation of lysine. This separation can be successfully accomplished in the presence of impurities such as heavy metal cations, inorganic anions, and aliphatic and aromatic amines. The limit of detection of lysine was 0.5μg per zone. The method has been used for successful in identification of lysine in Astymin (forte) and Alamine (forte) capsules.

Chapter-3

A novel micellar thin layer chromatographic method for simultaneous separation and on-plate identification of B-group vitamins along with

ascorbic and folic acids has been developed. A hybrid mobile phase constituting a mixture of 4% aqueous sodium dodecyl sulphate (SDS) + acetonitrile 1:2 (v/v) was identified as the most favorable for the resolution of multicomponent mixture of vitamins on silica high performance TLC plates (silica gel $60F_{254}$ catalog no. 1.05554,Merck, Germany). The resolved spots were identified by visualization under ultraviolet radiation (λ=254) in a closed UV- cabinet. The effects of type of sample solvent, concentration of surfactant (sodium dodecyl sulphate), volume ratio of acetonitrile in the mobile phase and the presence of essential amino acids in the sample have been examined. This method is simple, precise, sensitive and useful for the analysis of vitamins B_1, B_2, B_6, B_{12}, ascorbic and folic acids present in marketed pharmaceutical formulation.

Chapter-4 Aqueous micellar bile salt, sodium deoxycholate (NaDC) solution as additive in acetonitrile in the ratio (1:5 v/v) was identified as the most favorable mobile phase for on-plate identification and resolution of three series of common sugars (disaccharide, pentose and hexose) on commercially available high performance silica gel plates. Effect of mobile phase composition and the presence of heavy metal cations as impurities in the analyte sample have been investigated to optimize experimental conditions for the separation. The lowest possible amount of all sugar on HPTLC plate has been determined at nanogram level. This method was successfully applied for identification and separation of sugars in

pharmaceutical formulations [cough syrup (Honitus), multivitamin syrup (Becozinc)] and biological matrix (human blood).

Chapter-5 Thin-layer chromatographic procedures are described for the analysis and separation of a variety of organic molecules (amino acids, B-complex vitamins, purines and pyrimidines, neutral lipids, polar sugars and dyes) using water-in-oil-microemulsion containing N-cetyl-N, N, N trimethyl ammonium bromide (CTAB) as mobile phase. High performance thin layer chromatographic plates of silica gel G and cellulose were used as stationary phases. The proposed method is suitable for selective separation of bromocresole green from other dyes on cellulose HPTLC plates. Furthermore, vitamins (thiamine, pyridoxine and riboflavin in marketed formulations) were successfully resolved on silica HPTLC plates. Effect of other biomolecules as impurities was investigated on simultaneous separation of thiamine, pyridoxine riboflavin from their mixtures. The well intense colored compact spots for all compounds under study were realized.

Chapter-6 A novel high-performance thin layer chromatographic method has been developed for the resolution of five-coexisting nucleobases (adenine, guanine, cytosine, thymine, and uracil). The nucleobases were separated on aluminum-backed cellulose 60 F_{254} plates with the aid of 5.0% aqueous sodium deoxycholate (NaDC)-acetonitrile (AcN), 1:3 (v/v) as mobile phase. All the nucleobases were viewed on HPTLC plates under 254nm UV light. The order of R_F value given in parentheses was guanine

(0.12) < adenine (0.44) < cytosine (0.50) < uracil (0.72) < thymine (0.84). The effect of pH (acidity or basicity) of the mobile phase on the retention of individual nucleobases was examined. Furthermore, the effect of interference of mono- (Li^+, Na^+), and bivalent (Mg^{2+}, Ba^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}) cations; mono- (Br^-, CH_3COO^-, NO_3^-) , and bivalent (CO_3^{2-}, IO_4^-, SO_4^{2-} MoO_4^{2-}) anions, and complexing ligands (urea, and EDTA) on the retention behavior of nucleobases were also assessed. The chromatography of nucleobases was also performed on silica 60 F_{254}, RP-18 F_{254}, and kieselgel 60 F_{254} HPTLC plates. These TLC plates failed to separate the coexisting purines and pyrimidines. The detection limit of all nucleobases on cellulose 60 F_{254} layers was 5.4×10^{-2} μg $spot^{-1}$. The proposed method is rapid, easy, and reliable. It can be applied for routine analysis of DNA, and RNA nucleobases.

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DEPARTMENT OF APPLIED CHEMISTRY
Z.H. COLLEGE OF ENGINEERING & TECHNOLOGY
ALIGARH MUSLIM UNIVERSITY, ALIGARH-202 002 (INDIA)

Dr. Ali Mohammad
Ph.D., D.Sc. (F.N.A.Sc.)
Editor: Chemical & Environmental Research

Off. : 0571-2700920-23 Ext. 3000
Resi : 0571-2408196
University Fax : (0571) 2400528
E-mail : mohammadali4u@rediffmail.com

Certificate

This is to certify that the work embodied in this thesis entitled, **"Analytical Studies of Organic Compounds of Pharmaceutical Importance"** is the original contribution of **Ms. Sameen Laeeq**, carried out under my guidance and supervision, and it is suitable for the award of degree of **Doctor of Philosophy in Applied Chemistry** from Aligarh Muslim University, Aligarh.

16/4/2010

ALI MOHAMMAD
(Supervisor)

DEDICATED
TO MY
BELOVED PARENTS

Acknowledgements

First of all, I prostrate in reverence before ***Almighty Allah,*** *the benevolent, the cherisher and sustainer. I am highly indebted and grateful to my Lord "Almighty" who blessed me with innumerable favors to complete this academic work and its bringing in this present form.*

Completing a thesis is a challenge, thanking all those who contributed to it are an even greater one.

I would like to express my gratitude to my supervisor, Prof. Ali Mohammad, whose expertise, understanding, and patience, added considerably to my research experience. His truly scientist intuition has made him as a constant oasis of ideas and passions in science, which exceptionally inspire and enrich my growth as a student, a researcher and a scientist want to be. I am indebted to him more than he knows. Above all and the most needed, he provided me unflinching encouragement and support in various ways. I would also like to acknowledge all the members of the Department of Applied Chemistry Department for being helpful to me during my research plan.

Many thanks go in particular to Dr. Hina Shahab, Dr. Abdul Moheman, Dr. Showkat Ahmad Bhawani, Mrs. Almas Zehra, Dr. Shumaila Khatoon, Mr. Nazrul Haq, Ms. Fauzia Farooqi and Ms Asma, for their valuable advices and their willingness to

share their bright thoughts with me, which were very fruitful for shaping up my ideas and research.

Where would I be without my family? My parents deserve special mention for their inseparable support and prayers. My Father, Mr. Laeeq Ahmad, in the first place is the person who put the fundament my learning character, showing me the joy of intellectual pursuit ever since I was a child. My Mother, Mrs Waseem Jahan, is the one who sincerely raised me with her caring and gently love. My Younger Brother, Mohammad Yasir Laeeq, thanks for being supportive and caring. Last but not the least my in-laws, especially Mr. Rais Ahmad Khan for his love and affection.

My friends deserve my sincere expression of thanks namely Rakshi, Nasheed, Batool, Madiha, Deeba, Sara, Preena and all my batchmates of MSc.

I like also to express thankfulness to all the non-teaching Staff for their help and support during my research tenure.

Finally, I would like to thank everybody who was important to the successful realization of thesis, as well as expressing my apology that I could not mention personally one by one.

Sameen Laeeq

List of Publications

1. Mixed Surfactants Enable Separation of Lysine from Other Essential Amino Acids in TLC on Silica Gel; A. Mohammad, **S. Laeeq**, *J. Planar Chromatogr. –Mod. TLC* **20** (2007) 423.

2. Identification and Simultaneous Separation of Six Hydrophilic Therapeutic Vitamins by Micellar Thin Layer Chromatography; A. Mohammad, **S. Laeeq**, *The Icfai University, J. Chemistr.* **1** (2008) 19.

3. Application of Water-in-Oil Microemulsion for Chromatographic Study of Different Groups of Organic Compounds; A. Mohammad, **S. Laeeq**, *Der Pharm. Chemi.* **2** (2010) 281.

4. Identification of Coexisting Pentose, Hexose and Disaccharides with Preliminary Separation through Hydrophilic Interaction on Silica HPTLC Plate Using Aqueous Sodium Deoxycholate–Acetonitrile Mobile Phase System; A. Mohammad, **S. Laeeq,** *J. Liq. Chromatogr. Related Technol.* **(Communicated).**

5. Sodium Deoxycholate Micelles Activated Separation of Coexisting Five-Nucleobases By High-Performance Thin-Layer Chromatography; A. Mohammad, **S. Laeeq,** *J. Bioanal. Biomed.* **(Communicated).**

CONTENTS

1.1 INTRODUCTION

Analytical chemistry pertains to the determination of the chemical composition of matter. It is a multidisciplinary branch of science wherein a large number of research workers have contributed to its development [1]. No other branch of science finds so many extensive applications as analytical chemistry purely for two reasons: Firstly, it finds numerous applications in various disciplines of chemistry such as inorganic, organic, physical and biochemistry and secondly it finds wide applications in other fields of related sciences such as environmental science, agricultural science, biomedical and clinical chemistry, solid state research and electronics, oceanography, forensic science and space research. However, the identification of a substance, the elucidation of its structure and quantitative analysis of its composition are the aspects covered by modern analytical chemistry. For instance, most of the chromatographic methods were invented by biochemists, or biological scientists, while methods like nuclear magnetic resonance and mass spectrometry were discovered by physicists.

Analytical methods are the fundamental tools of the analyst. These classical methods of analyses have dominated the scene of analysis for the past few decades. Fortunately, with the discovery of modern methods of analysis, mainly involving instruments, these methods have been relegated. Nevertheless, these methods will not be phased out in spite of the greater advancement of newer methods of analysis for the simple reason that the new methods have their own limitations. They cannot be applied if the substance is present in large concentration and further in order to standardize the newer methods it is absolutely essential to use classical (gravimetric or volumetric) methods of analysis. According to the type of process used to perform the analysis, methods used for chemical analysis can be categorized as given in **(Figure 1.1)**. In the present circumstances when speed, simplicity, selectivity and sensitivity of analysis are of utmost importance, it is better to categorize such methods as modern methods of analysis instead of instrumental methods of analysis.

1.2 SEPARATION PROCESSES

A separation process is a physical or chemical method to transform a mixture of substances into two or more distinct products that differ in their chemical or physical properties. For either preparative or analytical purposes there are several methods used in chemistry to purify substances or to isolate them from other substances. There are many types of separations based on a variety of properties of materials. Among the most commonly used properties are those involving solubility, volatility, adsorption, and electrical and magnetic effects, although others have been used to advantage. The most efficient separation involves the conditions for which the differences in properties between two substances undergoing separation are at a maximum. There are three factors of importance to be considered in all separations: (a) the completeness of recovery of the substance being isolated, (b) the extent of separation from associated substances, and (c) the efficiency of the separation.

In industrial applications the ultimate goal is the isolation of a product of high purity, whereas in analysis the primary goal is the determination of the amount or concentration of substance in a sample.

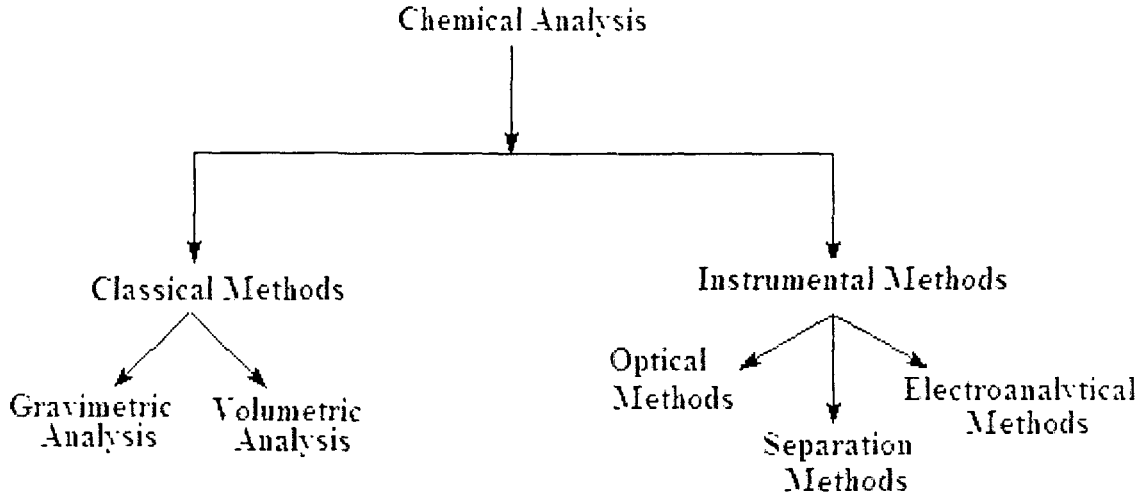


Figure 1.1: Major categories of chemical analysis

1.3 CHROMATOGRAPHY

What is chromatography? Simply, it is a broad range of physical methods used to separate or to analyze complex mixtures. The components to be separated are

distributed between two phases: a *stationary phase* bed and a *mobile phase* which percolates through the stationary bed.

Chromatography derived from Greek word χρώμα: *chroma* means color and γραφειν:*graphein* means to write) represents a collective term for a set of qualitative and quantitative techniques for the separation of mixtures. It involves passing a mixture dissolved in a "mobile phase" through a *stationary phase*, which separates the analyte to be measured from other molecules in the mixture based on differential partitioning/ sorption between the mobile and stationary phases.

The mobile phase may be either a liquid or a gas, while the stationary phase is either a solid or a liquid. This phenomenon involves the partition of a solute between a solvent firmly bound to a polar stationary phase and a solvent (mobile phase) which is allowed to interact with two physically distinct entities. The techniques find use in the separation, purification and identification of compounds before quantitative analysis is taken up.

Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for further use (and is thus a form of purification). Analytical chromatography is done normally with smaller amounts of material and is for measuring the relative proportions of analytes in a mixture.

Since its invention, the chromatography has become an essential part of bio-chemical laboratories. Using this analytical technique, scientists can tell what chemical compounds are present in complex mixtures. A simple classification of chromatographic methods is summarized in **Table 1.1.**

Table 1.1: Classification of chromatographic methods

S. No.	Type of Chromatography	Examples
1.	Adsorption Chromatography	Column Chromatography, Thin Layer Chromatography, Gas Solid Chromatography.
2.	Partition Chromatography	Paper chromatography, Reversed-Phase Thin Layer Chromatography (TLC), Classical Liquid-Liquid Chromatography.
3.	Modified Partition (or Bonded Phase) Chromatography	High-Performance Liquid Chromatography (HPLC) and High Performance (HP) TLC.
4.	Ion-exchange Chromatography	Cation and Anion Exchange Chromatography.
5.	Exclusion Chromatography	Ion-Exclusion and Gel Permeation Chromatography, Molecule Sieve Chromatography.
6.	Electrochromatography	Capillary and Zone Electrophoresis.

The identification and separation of various species can be achieved by an array of systematic procedures. Among the most versatile analytical separation techniques, chromatography has wider applicability. In spite of the popular belief and general acceptance of the contribution of *Tswett* as being the real discoverer of chromatography (literally "colour writing" from the Greek), the starting of chromatography predated to the work of *F. F. Runge* who investigated the separation of coloured substances (i.e. dyes) on paper [2] The work carried out by *Goppelsroeder* [3] and *Schonbein* [4] on chromatographic separation of substances on filter paper has been included in a report published by *Fischer* and *Schmidner* [5] in 1892. However, the concept of separation on column may be attributed to *Reed's* work, which was followed by *Day* who separated petroleum fractions with the help of columns [6,7]. The paper published in 1906 by *M. Tswett* a Lecturer of Botany at the University of

Warsaw provided the first description in nearly modern terms of chromatographic separation [8]. He described the resolution of different components of pigments as coloured bands on a calcium carbonate column like spectrum of light rays and termed it as *"chromatogram"*. The actual importance of *Tswett's* work remained dormant until about 1931, when separations of plant carotene pigments were reported by prominent organic chemist *Kuhn* [9,10]. His research attracted much attention and adsorption column chromatography became invaluable tool in the field of natural product chemistry. In 1941, *Martin* and *Synge* [11,12] laid another milestone in development of chromatography by reporting their discovery of liquid-liquid partition chromatography. One liquid was used as adsorbent and another liquid was allowed to percolate through the former, thus making the technique as a chromatographic process. This work initialized the development of other forms of chromatography.

1.4 THIN LAYER CHROMATOGRAPHY (TLC)

TLC is a subdivision of liquid planar chromatography in which the mobile phase; a liquid migrates by capillary action through the stationary phase which is in the form of a thin layer on an inert support. Components of a mixture are separated by distributing between mobile phase and stationary phases. Difference in the affinity of individual components to stationary and/or mobile phase facilitates their separation.

History of TLC

History of TLC has been reviewed by *Stahl* [13] *Kirchner* [14,15] and *Pelick et. al* [16]. The beginning of TLC can be ascribed to the report of Dutch biologist, *Beyerink* [17], who separated hydrochloric and sulphuric acids in the form of fine rings on thin layer of gelatin using a visualizing agent. Following the same method, *Wijsman* [18] identified in the presence of two enzymes in malt *diastase* using a fluorescent method for detecting separated enzymes on thin layer. He used the bacteria obtained from sea water as fluorescent agent. However the invention of TLC is usually credited to two Russian Scientists, *N. A. Izmailov*

and *M. S. Schraiber*, who used binder free horizontal thin layers (2 mm thick) of alumina spread on glass plate to the analysis of pharmaceutical preparations which led to the publication of their classical paper [19] on *"A Spot Chromatographic Method of Analysis and its Application in Pharmacy"* in 1938. Since their method consists of depositing a drop of sample solution being investigated and the development by the application of several drops of solvent on flat surface of adsorbent before observing the separated zones, it was called *"Drop Chromatography or Spot Chromatography"*. They also pointed out the usefulness of this method for preliminary testing of sorbent properties before their utilization in the form of column. Though *Izmailov* is best known for his fundamental work on TLC, his main field of interest was electrochemistry for which he received the *Mendeleiv* Prize of the Academy of Science of USSR in 1961.

In 1940, *Lapp* and *Erali* used a loose layer of alumina spread on a glass slide that was supported on an inclined aluminium sheet [20]. It is interesting that, in 1949, two American Chemists, *Meinhard* and *Hall* [21] gave the concept of *"Surface Chromatography"* and described their work on the use of microscope slides coated with a mixture of alumina (an adsorbent) and celite (a binder) to separate Fe^{2+} and Zn^{2+}. Their work was probably the first application of TLC for the separation of inorganic ions. A major breakthrough in the field of TLC came in the early 1960's with the availability of precoated plates [22]. The next major advance was the advent of HPTLC (High performance Thin Layer Chromatography). In 1973 *Halpaap* was one of the first to recognise the advantage of using a smaller average particle size of silica gel (about 5–6 mm) in the preparation of TLC plates. He compared the effect of particle size on development time, R_F values and plate height [23].

Commercially the plates were first called ''nano-TLC'' or ''HPTLC". In 1977 the first major HPTLC ''HPTLC high performance thin-layer Chromatography'' edited by *Zlatkis* and *Kaiser* [24]. In 1982 *Jost* and *Hauck* reported an amino (NH_2–) modified HPTLC plate, which was soon followed by cyano-bonded (1985) and diol-bonded (1987) phases [25-27]. In recent years TLC/HPTLC

research has entered the chiral separation field using a number of chiral selectors and chiral stationary phases.

Some of the differences between TLC and HPTLC are compared in **Table 1.2**. Automated multiple development (AMD) made its appearance in 1984 due to the pioneering work of *Burger* [28]. This improvement enabled a marked increase in number and resolution of the separated components. Numerous publications on TLC/HPTLC applications attest to the versatility and applicability of this technique in all branches of science. It has opened new fields of exploration and become an invaluable aid to separation scientists. It had recently been realized that modern high performance thin chromatography (HPTLC) initiated in 1975, rivals high pressure liquid chromatography (HPLC) and gas chromatography (GC) in its ability to resolve complex mixtures and to provide analyte quantification.

Table 1.2: Comparison of TLC and HPTLC

Parameter	TLC	HPTLC
Plate size	20×20 cm	10×20 or 10×10 cm
Average particle size	20 μm	5 μm
Adsorbent layer	100-250 μm	200 μm
Thickness	-	-
Plate height	30 μm	12 μm
Sample volume	1-5 μl	0.1-0.2 μl
Solvent migration	10-15 cm	3-6 cm
Distance	-	-
Separation time	30-200 min	3-6 min
Sample per plate	10	18 or 36
Diameter of separated spots	6-15 mm	2-6 mm
Detection limits	-	-
(a) Adsorption	1-5 ng	0.1-0.5 ng
(b) Fluorescence	0.05-0.1ng	0.005-0.01 ng

HPTLC provides faster separation, reduced zone diffusion, better separation efficiency and higher sensitivity.

At the present time all steps of the TLC process can be computer controlled. The use of highly sensitive charge coupled device (CCD) cameras has enabled the chromatographer to electronically store images of chromatograms for future use (identity or stability testing) and for direct entry into reports at a later date.

1.5 LATEST DEVELOPMENT IN TLC

After the pioneering work of Kirchner and Stahl, TLC became important for the separation of sample not amenable to analysis by GC. The rapid growth of TLC was slowed down during 1970s with the corresponding rise in popularity of HPLC. The capacity factors in HPLC are more reproducible than R_F values in TLC. However, recent improvements in TLC have removed many of its limitations **(Table 1.3).**

As a result of the recent innovations, several sub- techniques such as High Performance Thin Layer Chromatography (HPTLC), Overpressurised Thin Layer Chromatography (OPTLC), Centrifugal Layer Chromatography (CLC) and Reversed- Phase Thin Layer Chromatography (RPTLC), Radial Chromatography, Hot Plate Phromatography, Pyrolysis and TLC, Bioautography, Immunostaining and Enzyme Inhibition techniques came into light.

Table 1.3: Chromatographic differences between TLC and HPLC

	TLC	HPLC
Transport of eluent by	capillary	pressure
Presence of binder	yes / no	no
Pre-wetted stationary phase	no	yes
Influence of vapor phase	yes	no
Parallel samples	yes	no
Eluent with detection	no	yes
High efficiency	no	yes

1.6 TLC PROCEDURE

The TLC process is an off-line process in which all the procedural steps (sample preparation, sample application, development, drying of chromatogram and detection) are carried out independently **(Figure 1.2)**. The basic TLC procedure involves the spotting of sample mixture (1-2 µl) at about 2 cm above the lower edge of the TLC plate, drying the spot at room temperature. Develop the plate in a suitable mobile phase to a distance of 10-12 cm by one dimensional ascending technique in a cylindrical or rectangular closed chamber, withdraw the plate from the developing chamber, and dry the layer at 60°C in an electrically controlled oven for 30 min. Detect the spots on TLC plate using suitable reaction reagent and then measure the R_F values of the resolved spots. The differential migration of components results due to varying degrees of affinity of the components in a mixture for stationary and mobile phases.

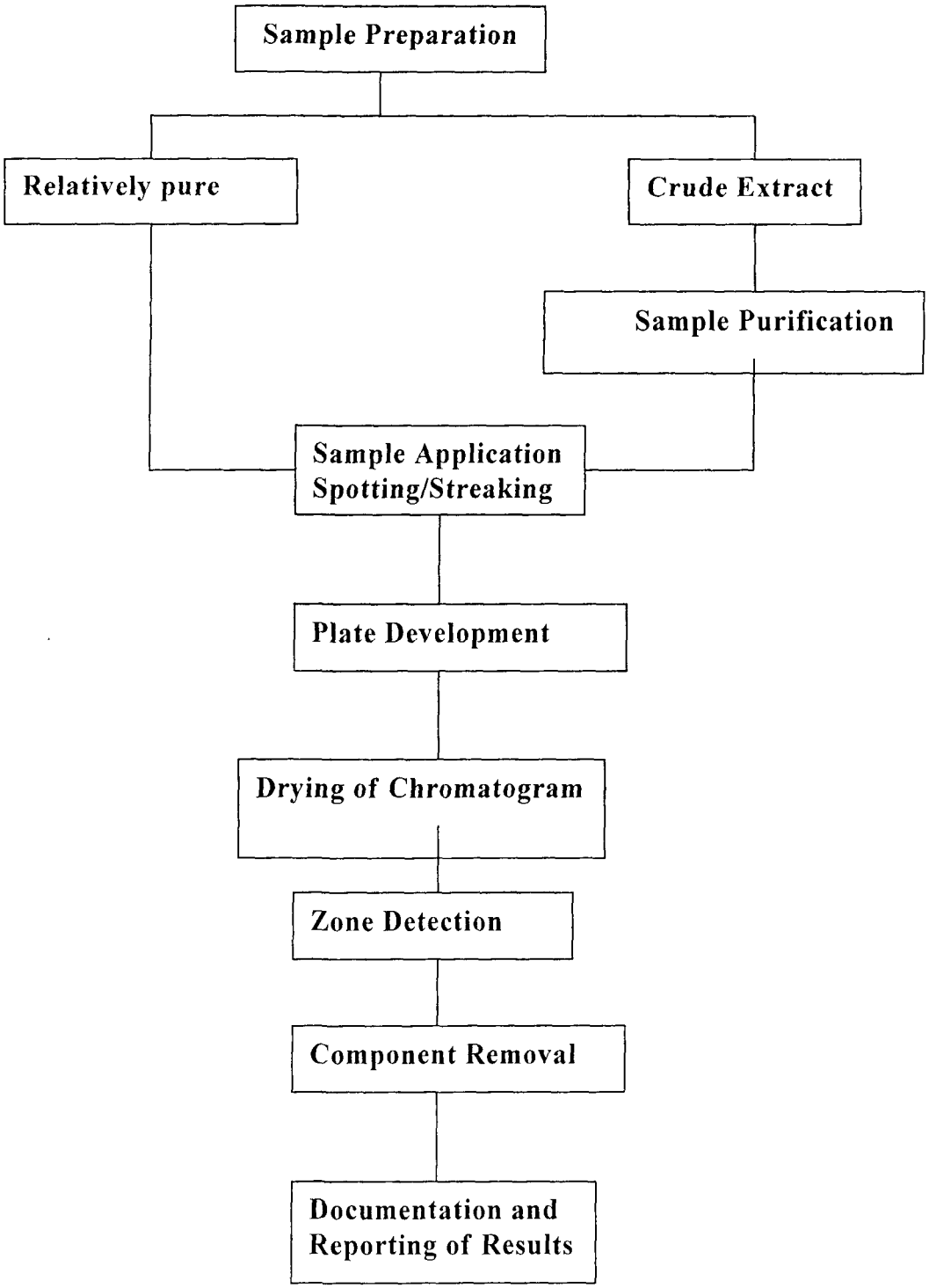


Figure 1.2: Scheme of typical thin layer chromatographic process

1.6.1 *Sample preparation*

Standard methods for sample preparation identification and separation of analyte present in a variety of samples such as plants, food, biological, geological and environmental samples have been reported. In general, solutions of the amino acids are prepared by dissolving appropriate weights in double distilled water to give concentrations of 1% (1 g/100 ml).

1.6.2 *TLC plate preparation or coating procedures*

The manual preparation of layers involves the coating of slurry of the adsorbent (silica gel, alumina and kieselguhr) on glass, aluminium or plastic sheet (20 cm x 20 cm or 20 cm x 10 cm) with the help of TLC applicator. The plates should be dried at room temperature followed by activation at 100°C in an oven and then used. The thickness of dried layer for analytical purposes is kept to 0.2-0.3 mm. A binder (starch, gypsum, dextrin or polyvinyl alcohol) is usually added to the layer material to provide better adhesion mechanical stability and durability.

1.6.3 *Sample application*

Sample application is one of the most important steps in the technology of TLC. Improperly applied samples result in poor chromatograms. Sample can be applied as spot or streak using micropipette, microsyringe, melting point capillaries etc. A number of automatic spotters of varying design are available for sample application. About 1 µl of 1% solution of the sample is applied on to a coated glass plates. The sample should be completely dried before placing the plate in the developing chamber.

1.6.4 *Development modes*

The process of migration of mobile phase through the sorbent layer to effect separation of the sample substance is called development. Ascending development has been the most commonly used mode of development in TLC. Other development modes such as multiple, circular and anti-circular, stepwise, continuous, two-dimensional, gradient, centrifugal and reversed-phase partition

development have also been used to limited extent. The distance for the migration of mobile phase has been kept to 10-12 cm for conventional TLC. While performing the development one should take care of the angle of the development and saturation of chamber apart from other factors.

1.7 TLC SYSTEMS

It comprises a combination of stationary and mobile phases. The proper selection of stationary and mobile phase condition decides the degree to which effective separations of components in a mixture can be achieved.

1.7.1 *Stationary phase (layer sorbent)*

The criterion to select a proper layer sorbent or adsorbent involves the following considerations:

- It should not chemically interact with the analyte.
- Color of the adsorbent should not interfere with chromatogram.
- It should be insoluble in solvent (i.e. mobile phase).
- Non catalytic (should not catalyze the decomposition of the substance).
- Physical and chemical properties of adsorbent should not change under experimental conditions.

In present study, we have utilized two commonly used adsorbents (silica gel and cellulose) as stationary phases; few words about these are worth mentioning.

Silica gel is an amorphous, porous substance and slightly acidic in nature. Binder is often incorporated into it to hold the adsorbent firmly on the plate. Thin layers of silica gel G (gypsum binder) and silica gel S (starch binder) with or without, fluorescent indicator have been used more frequently. At the surface of silica gel, the free valencies of the oxygen are connected either with hydrogen (Si-O-H. silanol groups) or with another silica atom (Si-O-Si, siloxane groups) **(Figure 1.3)**. The silanol groups represent adsorption-active surface centres that are capable to interact with solute molecules.

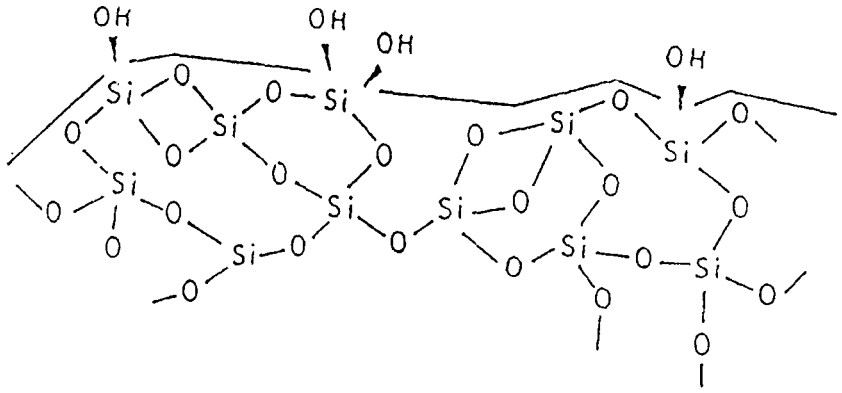


Figure 1.3: Structure of silica gel

Cellulose, an organic material is used as a sorbent in TLC when it is convenient to perform a given paper chromatographic separation by TLC with decreased development time and increase in the sensitivity of detection. Cellulose used for chromatography are composed of β-glucopyranose units, which are connected to one another at the 1, 4 positions **(Figure 1.4)**. Often cellulose thin layers need no binders because of the strong hydrogen bonding of the cellulose hydroxyl groups with the supports used.

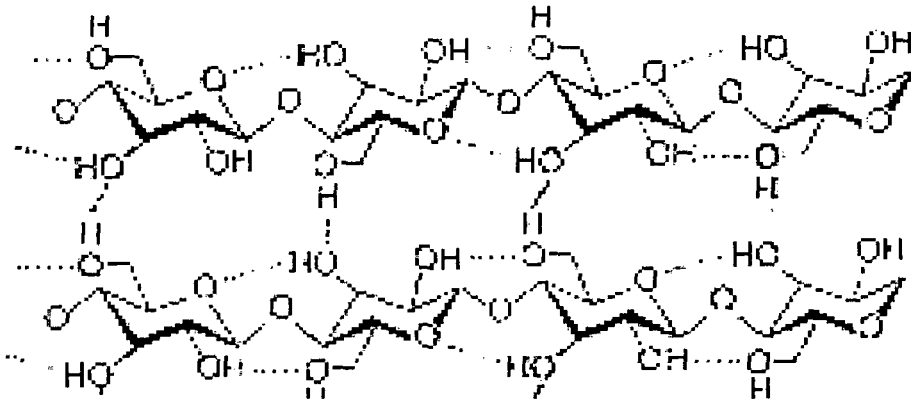


Figure 1.4: Structure of microcrystalline cellulose

Bonded sorbents as hydrophilic or hydrophobic modified sorbent phases are currently receiving wider acceptance. Reversed-phase TLC, in which the stationary phase is less polar than the mobile phase, was originally carried out on silica gel or kieselghur layers impregnated with a solution of paraffin, squalane, silicone oil, octanol, or oleyl alcohol. These phases are superior to those mentioned above under (a) and (b). The hydrophobic modified sorbents contain

organo- functional groups like methyl (RP-2), octyl (RP-8, dodecyl (RP-12), sorbents possesss amino-, cyno-, and diol residues as functional groups. The polar functional groups are bonded to the silica matrix via short- chain non-polar spacer. Impregnated plates of this kind require the use of aqueous and polar organic mobile phases saturated with the stationary liquid, and they cannot tolerate the use of non-polar organic solvents, which will strip the coating from the support.

1.7.2 *Mobile phase (solvent system)*

Selection of mobile phase depends on nature of the substances to be separated and adsorbent on which the separation is to be carried out. Separation possibility of complex mixture is greatly improved by the proper selection of mobile phase. Mobile phase should be as simple as possible and prepared from the purest grade of solvent. The use of mixtures composed of more than four components of mobile phase should be avoided because of problems associated with reproducible preparations. In contrast to mobile phases of higher volatility, which are capable to evaporate quickly from the sorbent layer, better reproducibility is achieved with mobile phases of lower volatility. The mobile phases used as developers in TLC may be categorized into following groups.

- Inorganic solvents: Solutions of mineral acids, bases, salts and mixture of acids, bases or their salts.
- Organic solvents: Acids, bases, hydrocarbons, alcohols, amines, ketones, aldehydes, organophosphates and their mixture in different proportions.
- Mixed solvents: Above mentioned organic solvents mixed with water, mineral acids, inorganic bases or dimethyl sulphoxide and buffered salt solution.
- Surfactant-mediated system: Aqueous and hybrid solutions of cationic, anionic and nonionic surfactants.

Presently, the interest of chromatographers is growing in identifying the green mobile phase system for future use. In our opinion aqueous solvent systems including pure water and aqueous solutions of amino acids, carbohydrates,

ethylene glycol, ethylene acetate and nonionic surfactants will occupy the prime position "Green Eluents" in chromatography.

1.8 VISUALIZATION

The process of detecting the spots on the plates after development is called visualization. Visualization of separated zones on TLC plate is performed by physical, chemical and biological methods.

- Physical methods of detection involve the use of spectroscopy (autoradiography), X-ray fluorescence and UV radiation etc. **(Figure 1.5).**

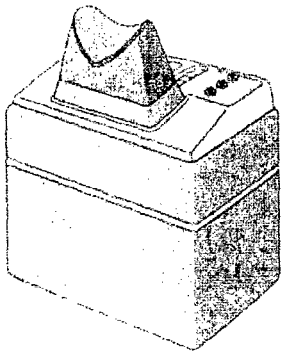


Figure 1.5: UV lamp with dark box

- Chemical detection methods involve the spraying of plates with a suitable reagent, which forms colored compounds with the separated species.
- Biological detection methods (bio-autography) are useful for specific detection of compounds with a certain physiological activity.

In addition to these techniques, enzyme inhibition, immunostaining and flame ionization detection methods have also been used.

1.9 IDENTIFICATION AND SEPARATION

In TLC the identification of separated compounds is primarily based on their mobility in a suitable solvent which is described by the R_F value of each compound. **Figure 1.6** shows a chromatographic plate.

$$R_F = \frac{\text{Distance of solute migration from the origin}}{\text{Distance of solvent migration from the origin}}$$

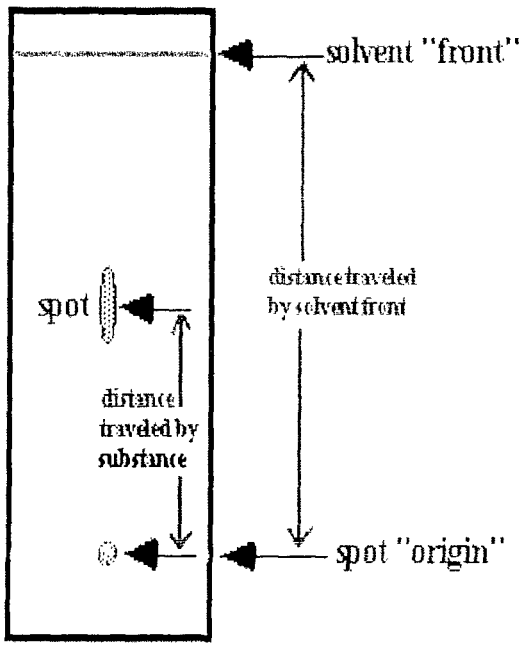


Figure 1.6: TLC plate showing distances traveled by the spot and the solvent after solvent front nearly reached the top of the adsorbent

R_F value ranges from 0.0 for a zone not leaving the point of application to 0.999 for zone migration with solvent front.

The R_F values in TLC are dependent upon many variables [29] which must be regulated carefully during the preparation and evaluation of the chromatogram, to obtain reproducible results. The major factors which influence the R_F values include:

- Nature of sorbent and mobile phase
- Layer thickness
- Room temperature
- Layer- activated temperature
- Chamber saturation
- pH of the medium
- Sample volume
- Relative humidity
- Mode of development technique

When two or more analytes have differential migration with the same chromatographic system, they are mixed thoroughly; the mixture is spotted on

the TLC plate and chromatographed. The separated components of the mixture are detected and their R_F values are recorded. Some of the basic requirements for a good separation are (a) each spot should be compact ($R_L - R_T < 0.3$), (b) the difference in R_F values of two adjacent spots should be at least 0.1, (c) no complexation should occur between/among separable species and (d) chromatography of individuals and the mixture should be performed under identical experimental conditions.

1.10 AMPHIPHILES

We have used amphiphilic molecules (i.e. surfactants) as eco-friendly components of mobile phase systems. It is therefore worthwhile to mention some of their important physic-analytical aspects.

The word **amphiphile** was coined by *Paul Winsor* 50 years ago. It is derived from two Greek words *amphi* means "double", "from both sides", "around" and the *philos* means affinity. An amphiphilic substance exhibits dual affinity. A typical amphiphilic molecule **(Figure 1.7)** comprise of two parts: a polar group with heteroatoms such as O, S, P,or N, included in functional groups (alcohol, thiol, ether, ester, acid, sulfate, sulfonate, phosphate, amine, amide etc.) and an essentially apolar group which is in general an hydrocarbon chain of the alkyl or alkylbenzene type, sometimes with halogen atoms and even a few nonionized oxygen atoms.The polar portion exhibits a strong affinity for polar solvents, particularly water, and it is often called hydrophilic part or hydrophile and vice-versa for apolar.

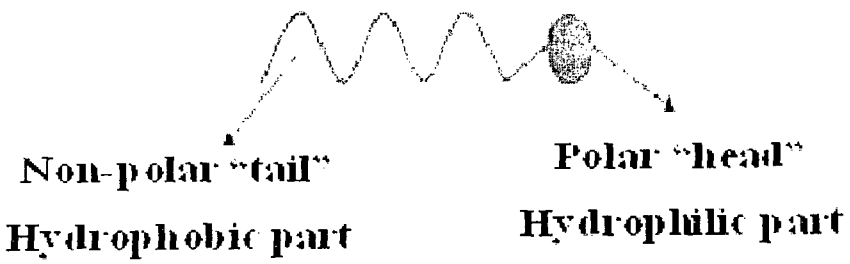


Figure 1.7: An amphiphilic molecule

1.11 SURFACTANTS

Surfactants are long chain amphiphilic organic or organometallic molecules containing a highly polar (hydrophilic or lipophobic) or "ionic head group" attached to a non-polar (hydrophobic or lipophilic) hydrocarbon tail of varying chain lengths. Surfactants, referred to as soaps, detergents, tensides, or surface active agents [30]. This dual functionality (amphipathic nature) is termed as lipophilic and hydrophilic tendencies. The combination of these two dissimilar chemical groups in a single substance is responsible for their surface active nature or ability to accumulate at an interface. These surface- active hydrocarbon molecules adsorbed at the oil/water interface by reducing the bare oil-water tension to low values. Because of this property, surfactants have been utilized **(Figure 1.8)** in many practical applications [31]. A list of some common surfactants is provided in **Table 1.4**.

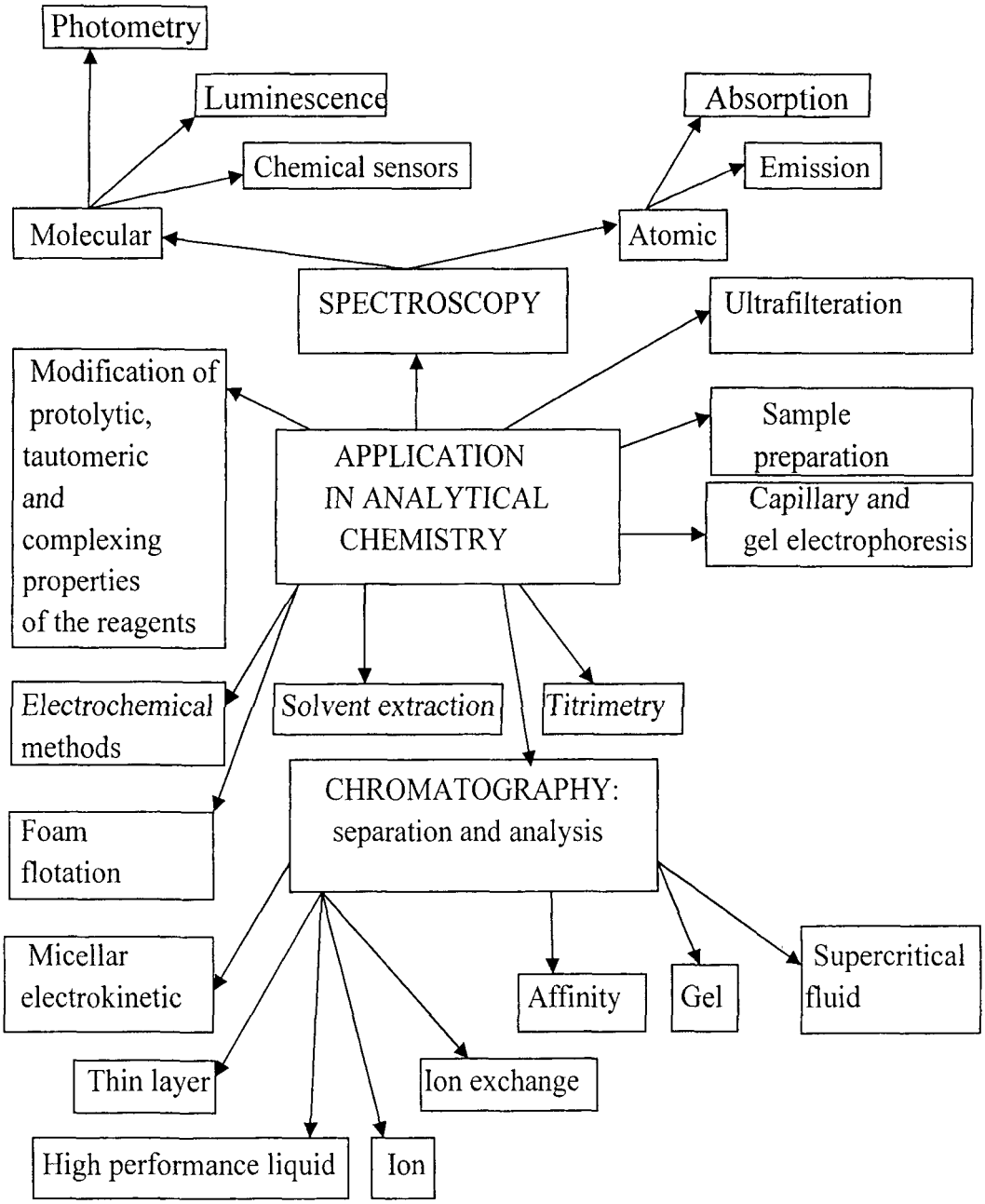


Figure 1.8: Fields of application of surfactants in analytical chemistry

The "head group" is either cationic (e.g. ammonium or pyridinium ion), anionic (e.g. hydroxy compounds) or zwitterionic (e.g. amine oxide, carboxylate or sulphonate betain) whereas the tail is hydrophobic and contain atleast 8 carbon atoms.
Depending upon the nature of hydrophilic group, surfactant can be classified as:

(a) Anionic surfactants

These surfactants bear a negative charge in the surface active portion [$R\text{-}X^-M^+$] and formed by the reaction product of an organic compound (high molecular weight acid or alcohols) with an inorganic compound (sodium hydroxide or sulfuric acids), wherein the surface active portion bears a negative charge. Alkali alkanoates or soaps, alkyl sulfates, aryl sulfonates and sulfonates of alkyl succinates are important classes of anionic surfactants. The most important anionic groups are carboxylate ($-CO_3^-$), sulfate ($-SO_3^-$) and sulfonate ($-SO_3^-$). They are high and stable foaming agents; however they do have the disadvantage of being sensitive to minerals and the presence of minerals in water (water hardness) or pH changes.

(b) Nonionic surfactants

These surfactants have hydrophobic/hydrophilic balance and hence there is neither a negative nor a positive charge on either part of the surfactant molecule [$R\ (OCH_2\ CH_2)]_m\ OH$. They do not ionize in aqueous solution, because their hydrophilic group is of a nondissociable type, such as alcohol, phenol, ether, ester, or amide. A large proportion of these nonionic surfactants are made hydrophilic by the presence of a polyethylene glycol chain, obtained by the polycondensation of ethylene oxide. They are called polyethoxylated nonionics.

(c) Cationic surfactants

The surface active portion bears a positive charge [$R\text{-}N^+\ (CH_3)_3\ X^-$] and are formed when alkyl halides react with primary or secondary or tertiary fatty amines.These surfactants are based upon quaternary nitrogen. Alkylammonium halides and tetra-alkylammonium halides are the most numerous in this class. When a single surfactant molecule exhibit both anionic and cationic dissociations it is

called ***amphoteric*** or ***zwitterionic***. This is the case of synthetic products like betaines or sulfobetaines and natural substances such as aminoacids and phospholipids.

(d) Biosurfactants

In addition to these types of micellar - forming surfactants, there is another class of molecules that can associate in water to form micellar helical aggregates known as the Bile Salts [32].

Bile salts are very important biological detergent-like molecules. However, they differ from the long-chain alkyl surfactants as they possess a hydrophobic and a hydrophilic face **(Figure 1.9)**. Consequently, bile salts exhibit a different type of aggregation behavior. This aggregation process is viewed as consisting of the stepwise formation of initial primary micelles which are composed of 2 - 8 monomers held together by hydrophobic interactions between the bile salt nonpolar faces. At higher bile salt concentration (or high ionic strength) , the primary micelles can further aggregate to form larger , rod - like cylindrically shaped secondary bile salt micelles because of intermolecular hydrogen bonding between their hydroxyl groups[33-34].

Table 1.5 presents the name, structure, and micellar parameters of some common bile salt. A recent multi-volume series lists the trade name, chemical name, manufacturer, form, properties, toxicity, composition, principal and secondary uses, etc. for many of these surfactants [35].

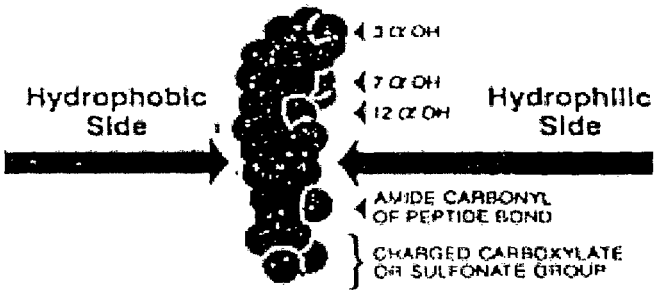


Figure 1.9: Model of a conjugated bile salt molecule (side view) which shows the spatial arrangement of the hydrophobic and hydrophilic face

Table 1.4: Some typical surfactants, formulae and their CMCs

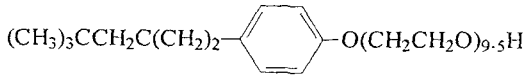
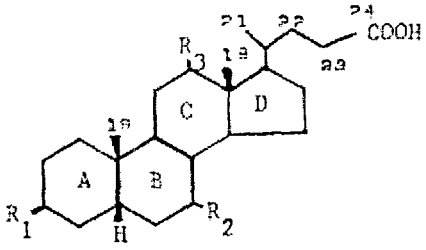
Surfactants	Formulae	CMC(M)
Anionic		
Sodium dodecyl sulfate (SDS)	$CH_3(CH_2)_{11}OSO_3Na^+$	8.1×10^{-3}
Potassium perfluoroheptanoate	$C_7F_{15}COO^-K^+$	3.0×10^{-2}
Sodium polyoxyethylene(12) - dodecyl ether	$CH_3(CH_2)_{11}(OCH_2CH_{12})_{12}OSO^-{}_3\ Na^+$	2.0×10^{-4}
Cationic		
Cetylpyridinium chloride	$C_{16}H_{33}N^+C_5H_5Cl^-$	1.2×10^{-4}
Cetyltrimethyl ammonium bromide (CTAB)	$CH_3\ (CH_2)\ _{15}N^+\ (CH_3)\ _3Br^-$	9.0×10^{-4}
Nonionic		
Polyoxyethylene (6) dodecanol	$CH_3(CH_2)_{11}(OCH_2CH_2)_6OH$	9.0×10^{-5}
Polyoxyethylene (23)- dodecanol (brij-35)	$CH_3(CH_2)_{11}(OCH_2CH_2)_{23}OH$	1.0×10^{-4}
Triton X-100		2.8×10^{-3}
Zwitterionic		
N-Dodecyl-N,N-dimethylammonium-3-propane-1 sulfonic acid (SB-12)	$CH_3(CH_2)_{11}N^+(CH_3)_2(CH_2)_3SO3^-$	3.0×10^{-3}
N,N-Dimethyl-N (carboxymethyl) octylammonium salt	$C_8H_{17}N^+(CH_3)_2CH_2COO^-$	25×10^{-2}
Nonaqueous (reversed)		
Bis(2-ethylhexyl) sodium sulfosuccinate (AOT)	$NaO_3SCH(CH_2COOC_8H_{17})COOC_8H_{17}$	6.0×10^{-4}

Table 1.5: Structure and micellar parameters of some bile salts[a]

Structure	Name	CMC, mM	N
			
if R_1=R_2=R_3=H;	Cholanoic acid	---	---
if R_1=R_2=R_3=0H;	Cholic acid (CA)/sodium cholate (NaC)	12.5[b]	3 [b]
if R_1=R_3=0H; R_2=H;	Deoxycholic acid (DCA)/sodium 6.4 deoxycholate (NaDC)	6.4[b] 2.8[C]	14[C]

[a]Derivatives of cholanoic acid. [b]In 0.001 M NaOH. [c]In 0.15 M NaCl.

1.12 MICELLES

The various structures formed in aqueous solution on increasing the concentration of surfactant are illustrated in **Figure 1.10**. They are

a) Rod like micelles
b) Spherical micelles
c) Cylindrical
d) Hexagonal
e) Lamellar micelles

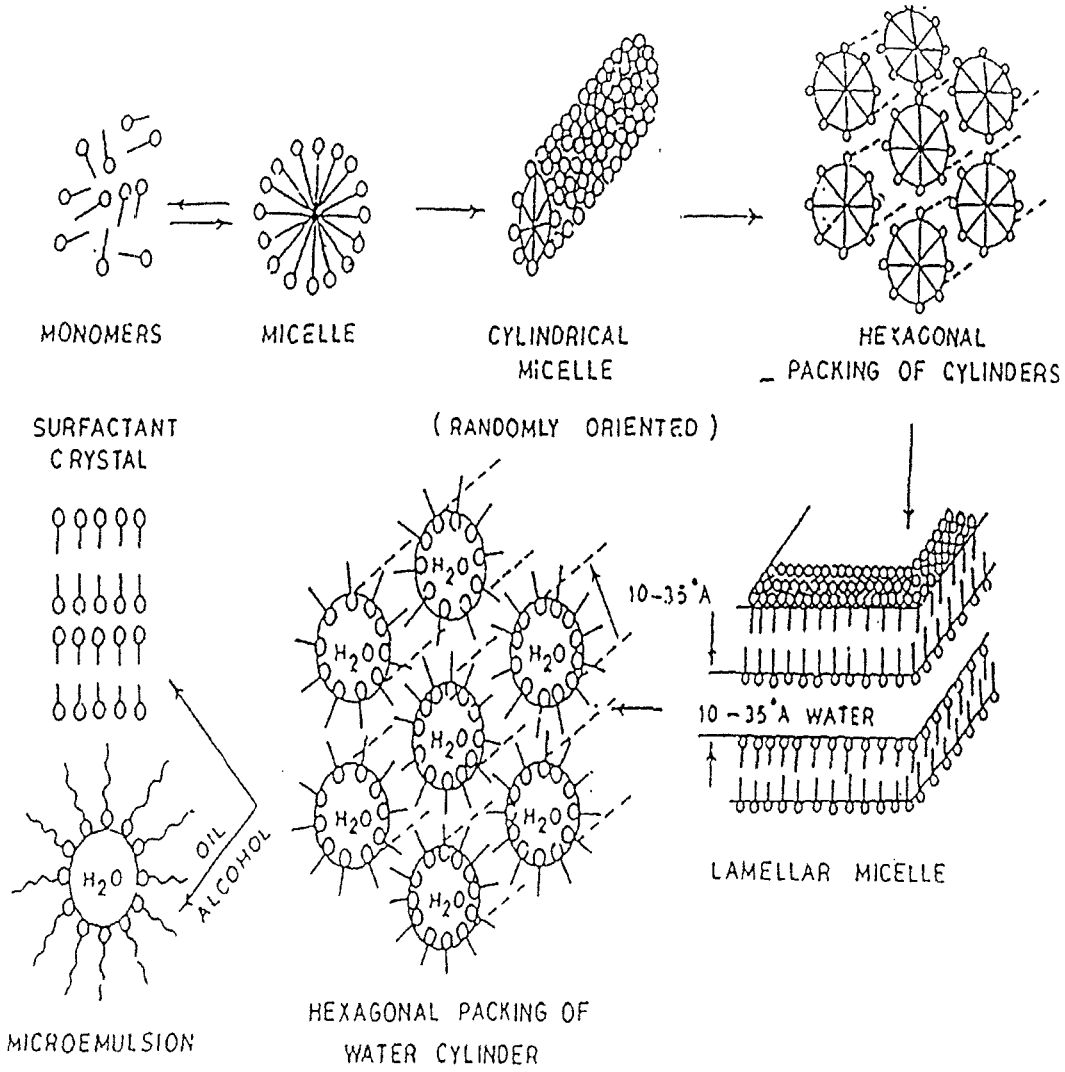


Figure 1.10: Representation of different shapes of micelles

There are mainly two types of micelles:

(a) Normal micelles

The molecular organization of surfactant molecules in aqueous solution results in the formation of normal micelles. Above CMC, the surfactant molecules are self aggregated in such a manner that the hydrophobic moieties (i.e. hydrocarbon tails) are oriented inward forming a non-polar core and hydrophilic (polar) head groups are outward keeping themselves in contact with the bulk aqueous phase **Figure 1.11 (a)**. Normal aqueous micelles are generally formed from singly-chain surfactants and chain branching inhibits micellization.

(b) Reverse micelles

In contrast to the normal micelles which are formed in polar (i.e. aqueous media) solvents, reverse micelles are formed in non-polar solvents like hexane or chloroform and a trace of water where the polar head groups of the surfactant are directed towards the interior of the aggregate and the hydrocarbon chains are in contact with the non-polar solvent **Figure 1.11(b)**. Reverse micelles offer the same potential advantages for analysis as do normal micelles. An interesting aspect of reverse micelles is their capability to solubilize water in the interior of micelle structure.

(a) **(b)**

Figure 1.11: Different types of micelles

1.13 PROPERTIES OF SURFACTANTS

At low concentrations, surfactants will favor arrangement at the surface. As the surface becomes crowded with surfactant, more molecules spontaneously aggregate themselves forming self-assemblies called micelles. This

concentration is called the Critical Micelle Concentration (CMC) [36]. The process of micelle formation is called micellization. **Figure 1.12** represents possible solubilization sites. A charged solute (A) would be electrostatically repelled from the micelle surface of the same charge (ionic micelle) while an oppositely charged solute (B) would be electrostatically attracted to the micellar surface. Nonpolar solutes (C) would partition to the outer part of the more hydrophobic core region. Amphiphilic solutes (D) would attempt to align themselves so as to maximize the electrostatic and hydrophobic interactions possible between it self and the surfactant molecules.

Micelles do not exist at all concentrations and temperatures. Upon introduction of surfactants into the system they will initially partition into the interface, reducing the free energy of system by (a) lowering the energy of the interface and (b) by removing the hydrophobic parts of the surfactant from contacts with water. Subsequently, when the surface coverage by the surfactants increases and the surface free energy (surface tension) has decreased, the surfactants start aggregating into micelles, thus again decreasing the system free energy by decreasing the contact area of hydrophobic parts of the surfactant with water. Upon reaching CMC, any further addition of surfactants will just increase the number of micelles. CMC is an important characteristic of a surfactant. Before reaching the CMC, the surface tension changes strongly with the concentration of the surfactant. After reaching the CMC, the surface tension becomes almost constant **(Figure 1.13)**. CMC is independent of interface and is therefore, a characteristic of the surfactant molecule. The average number of monomers per micelle is called the aggregation number (N). At 25°C and 1 atmospheric pressure, the CMC is typically less than 20 mM, with each micelle consisting of 40-140 monomers.

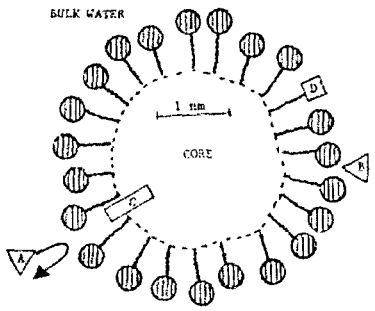


Figure 1.12: Simplified cross section of an aqueous normal micelle showing possible solubilization sites

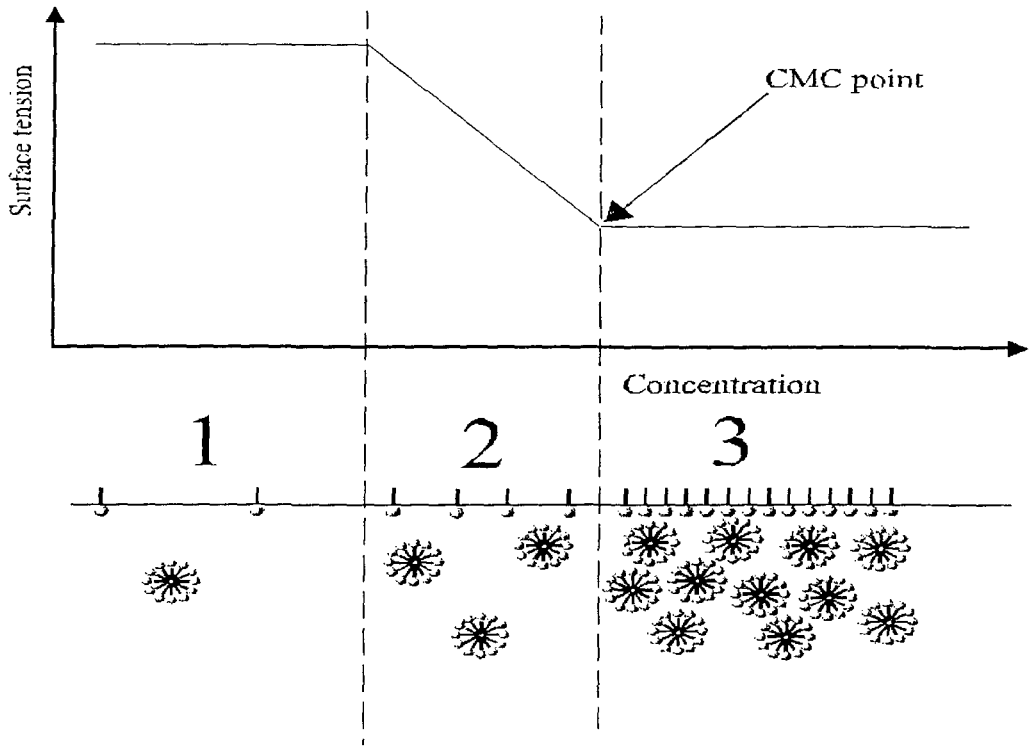


Figure 1.13: A graph of surface tension vs log of concentration of surfactant added will appear as follows

1.14 SURFACTANT-MEDIATED TLC SYSTEMS

The first intentional use o f surfactants in chromatography at concentrations above the CMC was proposed in 1977 by *Armstrong* and *co-workers* [37-49]. Since this initial report, Pseudophase Liquid Chromatography (PLC) or Micellar Liquid Chromatography (MLC), has moved from the realm of an academic novelty to a demonstrated practical separation technique as testified by

significant number of publications. **Figure 1.14** shows the growth in the number of reports on MLC since 1980 to present days. The basis for separation employing micellar mobile phases stems from their ability to differentially solubilize and bind structurally similar solutes.

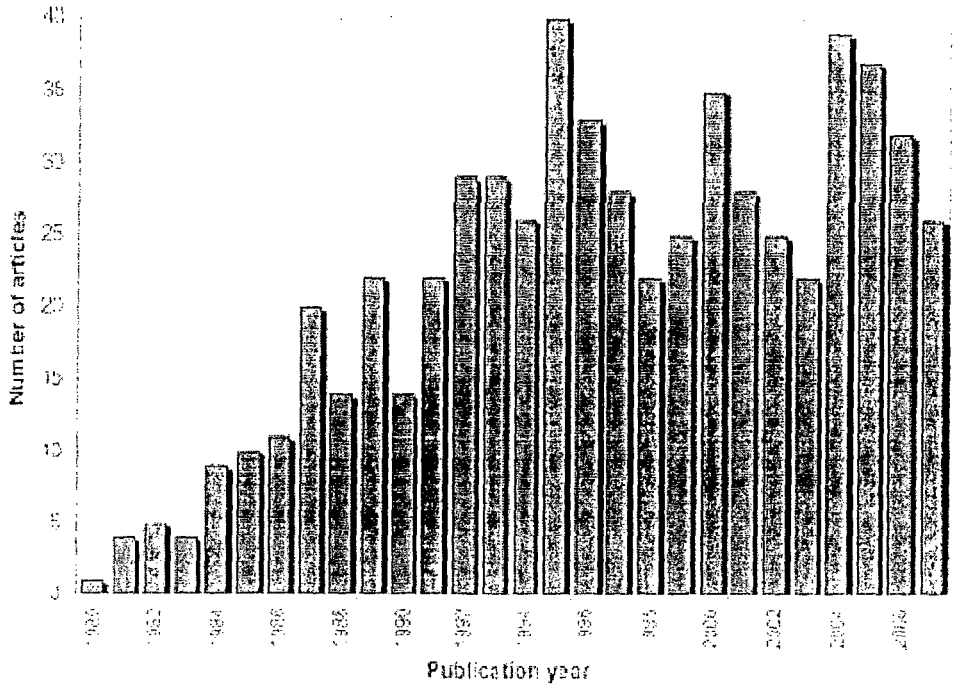


Figure 1.14: Publications dealing with MLC published over the 1980–2007 time period (612 articles found in the Elsevier, CAS and MedLine databases, Scopus and Sci Finder search engines on December 2007)

Since the work presented in this thesis is related to the use of surfactant – mediated mobile phase systems, it is worthwhile to describe briefly the behavior of surfactants in aqueous medium. The following paragraphs are devoted to highlight the utility of surfactant as eluents in chromatography.

Surfactant – mediated system contains surfactant as one of the components of mobile phase. Surfactant in the aqueous mobile phase can be used in the following ways:

(a) As monomer surfactants where the concentration of surfactant in aqueous mobile phase is restricted to well below the Critical Micelle

Concentration (CMC) of the surfactant. These mobile phases are most suited to separate ionic species by Ion Pair Chromatography (IPC).

(b) As surfactant micelles where the surfactant concentration is kept well above its CMC value. In such cases, the mobile phase is composed of surfactant molecules in the form of monomers and aggregates (or micelles). These mobile phases are very useful for simultaneous separation of ionic and non-ionic compounds by Micellar Liquid Chromatography (MLC).

(c) As microemulsion where surfactant in the presence of water, an oil (hydrocarbon) and co-surfactant (i.e. medium chain length amine or alcohol) is used as transparent solution. A brief description of microemulsions is given below.

It is well established that large amounts of two immiscible liquids (e.g. water and oil) can be brought into a single phase (macroscopically homogeneous but microscopically heterogeneous) by addition of an appropriate surfactant or a surfactant mixture. This unique class of optically clear, thermodynamically stable and usually low viscous solutions, called "Microemulsions" have been the subject of extensive research over the last two decades primarily because of their scientific and technological importance [50]. Microemulsions can have characteristic properties such as ultralow interfacial tension, large interfacial area and capacity to solubilize both aqueous and oil-soluble compounds [51-54].

Microemulsions can be prepared by controlled addition of lower alkanols (butanol, pentanol and hexanol) to milky emulsions to produce transparent solutions comprising dispersions of either Water-in-Oil (w/o) or Oil-in-Water (o/w) in nanometer or colloidal dispersions (~ 100 nm). The lower alkanols are called cosurfactants, they lower the interfacial tension between oil and water sufficiently low for almost spontaneous formation of the said microheterogeneous systems. The miscibility of oil, water and amphiphile (surfactant plus cosurfactant) depends on the overall composition which is system specific. Ternary and quaternary phase diagrams can describe the phase manifestations and are essential in the study of microemulsions.

The important applications of microemulsions include: enhanced oil recovery; fuels; coatings and textile finishing; lubricants, cutting oils and corrosion inhibitors; cosmetics; agrochemicals; food and pharmaceuticals; environmental remediation and detoxification; production of microporous media synthesis; liquid membranes. Microemulsion systems have been successfully applied in modifying chemical, photochemical, electrochemical and electrocatalytic reactions; analytical and bioseparations and polymerization processes.

There are two types of microemulsions (i) Oil-in-water and (ii) Water-in-oil

In terms of chromatographic applications, the advantages of employing surfactant micellar mobile phases includes, enhanced selectivity, low cost, low toxicity and the ability to simultaneously chromatograph both hydrophilic and hydrophobic solutes among others.

1.15 PHARMACEUTICALLY IMPORTANT ORGANIC COMPOUNDS

Biomolecules are complex organic molecules. These molecules form the basic structural constituent of a living cell. The organic compounds such as amino acids, vitamins, nucleotides and carbohydrates serve as building blocks of complex biomolecules.

A diverse range of biomolecules exist, including:

- Monomers:
  - **Amino acids**
  - **Nucleotides**
  - **Monosaccharides**

- Small molecules:
  - Lipids, phospholipids, glycolipids, sterols, glycerolipids
  - **Carbohydrates**
  - **Vitamins**
  - Hormones, neurotransmitters
  - Metabolites

- Polymers:
  - ❖ Peptides, oligopeptides, polypeptides, proteins
  - ❖ Nucleic acids, DNA, RNA
  - ❖ Oligosaccharides, polysaccharides (including cellulose)
  - ❖ Cellulose, lignin
  - ❖ Hemoglobin

Biological matrices are very complex; and therefore effective sample preparation must include particulate cleanup to provide the components of interest in a solution, free from interfering matrix elements, and in an appropriate concentration. Therefore thin layer chromatographic can be the most advantageous and simplest analysis techniques which we have used to identify four groups of organic compounds with preliminary separation. These compounds represented in bold letters were analysed by us using TLC as analytical technique. These following paragraphs are decoded to summarize main aspects of these compounds.

❖ Amino acids

These compounds contain carbon, hydrogen, oxygen and nitrogen. They serve mainly as monomers (building blocks) of proteins. An **amino acid** is represented by a general structure **(Figure 1.15).**

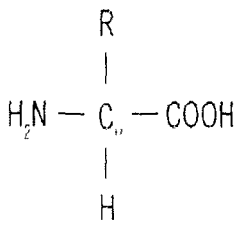


Figure 1.15: General structure of amino acid

They are colourless, crystalline solids, water-soluble high melting solids ,behave like salts and can exist as zwitterions at isoelectric pH (pI).The α-carbon atom has 'R' which is a side chain which is different for different amino acids

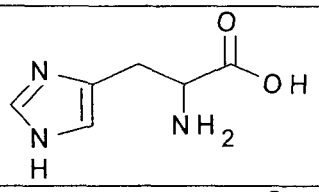
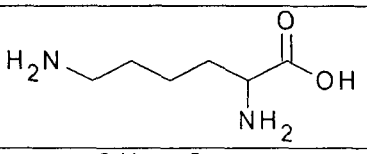
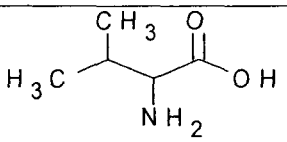
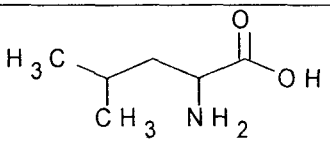
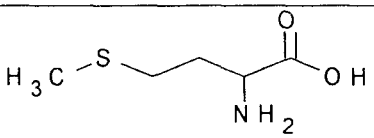
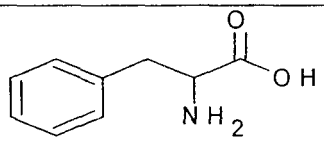
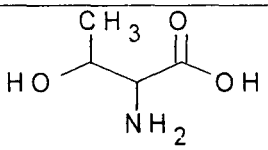
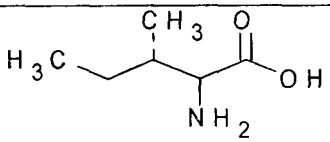
Though, 300 amino acids occur in nature, about 20 amino acids are found in proteins.

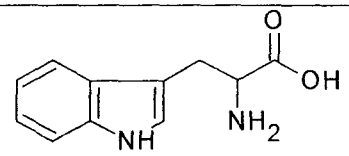
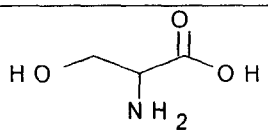
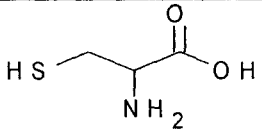
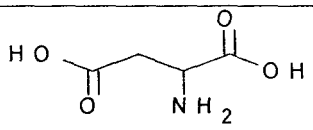
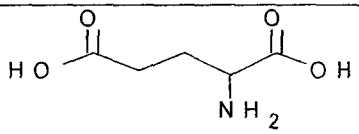
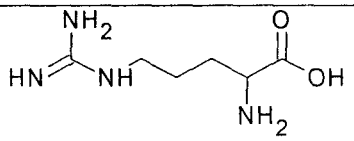
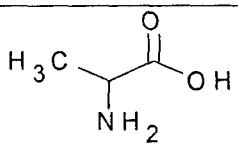
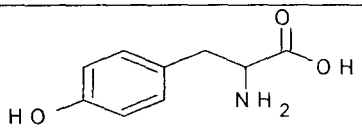
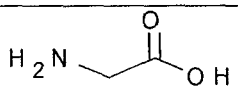
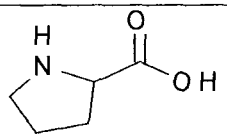
Amino acids may also be categorized as polar, non-polar, hydrophobic, hydrophilic, aliphatic and aromatic. Also they are classified as

(i) **Essential amino acids:** Their carbon skeleton cannot be synthesized by human beings and therefore, these amino acids are to be taken in food for normal growth.

(ii) **Non-essential amino acids:** All body proteins do contain all the non-essential amino acids. Their carbon skeleton can be synthesized by metabolic pathways and therefore their absence in the food will not adversely affect the growth **(Table 1.6).**

Table 1.6: Classification of amino acids on the basis of nutritional requirements

Essential amino acids	Three letter symbol	Structural formula	pI
Histidine	His		7.59
Lysine	Lys		9.74
Valine	Val		5.96
Leucine	Leu		5.98
Methionine	Met		5.74
Phenylalanine	Phe		5.48
Threonine	Thr		5.64
Isoleucine	Ile		6.02

Non essential amino acids			
Tryptophan	Trp		5.89
Serine	Ser		5.68
Cysteine	Cys		5.07
Aspartic acid	Asp		2.77
Glutamic acid	Glu		3.22
Arginine	Arg		10.76
Alanine	Ala		6.00
Tyrosine	Tyr		5.66
Glycine	Gly		5.97
Proline	Pro		6.30

❖ Vitamins

Vitamins are necessary to ensure healthy development and bodily functions in both children and adults. There are six different types of vitamins. Each serves a different purpose in our bodies. Vitamins are the organic compounds required

for the normal maintenance and health of an organism in minute quantities and their absence cause specific deficiency diseases. These are required in diet in order to perform specific biological functions. There are about 15 vitamins essential for humans. Plants can synthesize cell vitamins whereas only a few are synthesized in animals and hence need to be supplied in the foods. A human body needs substances like vitamins in order to develop and grow normally. There are around 13 that our body needs in order to function well. They are the A, C, D, E, K, and B (niacin, pantothenic acid, biotin, thiamine, riboflavin, folate, B_6, and B_{12}) are normally found in the food that we eat. However, people who practice a vegetarian diet may need to take B_{12} supplements. Vitamins are either water soluble or fat soluble. Fat soluble vitamins (A, D, E, and K) are stored in the fat tissues and in liver. Water-soluble vitamins represent a group of structurally and functionally unrelated compounds that share a common feature of being essential for normal health and well-being **(Table 1.8)**. They are quite different and do not get stored as much as fat soluble ones; instead, they travel through the bloodstream. Excess water soluble vitamins gets drained out though urination. Vitamins such as C and a large number of the B like B_1 (thiamin), B_2 (riboflavin), niacin, B_6 (pyridoxine), folic acid, B_{12} (cobalamine), biotin, and pantothenic acid are all water soluble and are necessary for our bodies to function properly.

Table 1.8: Vitamins their dosage, functions and sources

Vitamin	Dosage	Functions	Natural Sources
Vitamin A Retinol and/or Beta Carotene	5000 - 50,000 IU	Helps build healthy eyes, required for growth and bone development. Beta Carotene is a good antioxidant. Helps healing of infections.	Carrots, yams, pumpkins, yellow or orange fruits, beet greens, fish, eggs, tuna.
Vitamin B_1 Thiamine	25 - 300mg	Helps in carbohydrate metabolism and energy production. Required for normal nerve function.	Whole grains, rice bran, lean meats, fresh peas, beans, wheat germ, oranges, poultry, fish, enriched pastas.
Vitamin B_2 Riboflavin	25 - 300mg	Helps in production of energy from foods and the formation of red blood cells.	Fortified grains & cereals, leafy green vegetables, poultry, fish, yogurt, milk, cheese.
Vitamin B_3 Niacin	25 - 300mg	Assists in release of energy from carbohydrates, fats and proteins; helps promote healthy skin.	Fortified breads and cereals, brewer's yeast, broccoli, carrots, cheese, dandelion greens, dates, eggs, fish, milk peanuts, potatoes, tomatoes, tuna, veal, beef liver, chicken breast.
Vitamin B_5 Pantothenic Acid	10 - 300 mg	Helps release energy from foods; required for synthesis of many substances.	Lean meats, whole grain cereals, fish, legumes.
Vitamin B_6	2 - 300 mg	Essential for protein metabolism and nervous system function; participates in synthesis of hormones and red blood cells.	Whole grain breads and cereals, fish, chicken, bananas.

Vitamin B_9 Folic Acid	400 - 1,200 mcg	Essential for red blood cell formation and synthesis of DNA and protein.	Fortified cereals, pinto beans, navy beans, green leafy vegetables, beef, brown rice, bran, cheese, lamb, liver, milk, mushrooms, oranges, split peas, pork, tuna, whole grains.
Vitamin B_{12} Cyanocobalamin	25 - 500 mg	Helps maintain healthy nervous system, required for normal growth and for production of red blood cells. Helps breakdown fatty acids.	Ham, clams, cooked oysters, king crab, herring, salmon, tuna, lean beef, liver, low fat diary products.
Vitamin C	60 - 5,000 mg	Required for formation of connective tissue, bones and teeth; assists in utilization of other vitamins, acts as an antioxidant.	Citrus fruits, strawberries, broccoli, melons, peppers, collards, dandelion greens, onions, radishes, watercress.
Vitamin D	400 - 800 IU	Aides in normal bone growth and tooth function; facilitates calcium and phosphorus absorption.	Sun exposure, sardines, salmon, fortified milk, fortified cereals, herring, liver, tuna, margarine, cod liver oil.
Vitamin E	30- 1,200 IU	As an antioxidant it protects body cells and helps maintain normal red blood cells.	Whole grains, wheat germ, nuts, spinach, sunflower seeds.
Vitamin H Biotin	0.3 - 1 mg	Assists in metabolism of carbohydrates and synthesis of fats and proteins.	Legumes, nuts.
Vitamin K	80 mcg	Essential in the blood clotting process.	Green leafy vegetables like kale, spinach, broccoli, cauliflower.

❖ Carbohydrates

These are the organic molecules that are composed of elements carbon, hydrogen and oxygen. These carbohydrates are referred to as saccharides or sugars. Carbohydrates are defined as polyhydroxy-aldehydes or polyhydroxy ketones or compounds, which produce them on hydrolysis. They supply energy and serve as structural constituents. They have the general formula $C_x(H_2O)_y$. There are five major classes of carbohydrates **(Table 1.7)**.

- Monosaccharides
- Disaccharides
- Polysaccharides
- Oligosaccharides

Table 1.7: Monosaccharide classifications based on the number of carbons

Number of Carbons	Category Name	Examples
4	Tetrose	Erythrose, Threose
5	Pentose	Arabinose, **Ribose**, Ribulose, **Xylose**, Xylulose, Lyxose
6	Hexose	Allose, Altrose, **Fructose**, **Galactose**, **Glucose**, Gulose, Idose, **Mannose**, Sorbose, Talose, Tagatose
7	Heptose	Sedoheptulose

Sugars in bold we have used as analyte in our experiment

Functions of carbohydrates: Carbohydrates participate in a wide range of functions:

- Carbohydrates are most abundant dietary source of energy for all organisms.
- They supply energy and serve as storage form of energy.
- Carbohydrates such as glucose, fructose, starch, glycogen, etc. provide energy for functioning of living organisms.

❖ Nucleobases

A **nucleic acid** is a macromolecule composed of chains of monomeric nucleotides Nucleic acids were first discovered by Friedrich Miescher in 1871. Nucleotides are molecules that constitute the structural units of nucleic acids (e.g., DNA and RNA) and play central roles in many biochemical processes. These molecules serve as transmitters of genetic information and form structures within cells. Nucleic acids are universal in living things, as they are found in all cells and viruses. The five main natural nucleobases adenine, cytosine, guanine, thymine and uracil are involved in the self-assembly of one of nature's most interesting and intriguing class of biopolymers, namely the nucleic acids DNA and RNA. As such, these nucleobases have held a fascination to researchers in a diverse range of fields. Nitrogenous bases are found in nucleotides which are of two types:

(i) Purines

(ii) Pyrimidines

The purines and pyrimidines form an important part of the DNA and RNA which form the blueprint of genomes. The base pairing between the nucleotides result into the formation of important bonds required in chemical reactions. These are aromatic heterocyclic organic compounds which consist of the pyrimidine ring fused to a ring of imidazole. In the process of nucleotide synthesis, the purines and pyrimidines form hydrogen bonds between each other. The structure of the nucleotides is such that three hydrogen bonds are formed between guanine and cytosine while adenine and thymine form two hydrogen bonds with each other. This bonding which takes place between purines and pyrimidines is known as base pairing.

In addition the analysis of above mentioned pharmaceutically important organic compounds, some of important dyes have also been analysed by us using thin layer chromatographic separation technique. A short description of dyes is given.

❖ Dyes

A dye can be described as a colored substance that has an affinity to the substrate to which it is being applied. It is generally applied in an aqueous solution, and may require a mordant to improve the fastness of the dye on the fiber. Dyes are added to food or drinks to change their color and to improve their appearance and taste because people associate certain colors with certain flavors and the color of food can influence the perceived flavor.

The first synthetic dye commonly known as "Mauve" was prepared by *W.H. Perkin* in 1956.

Dyes can be classified on the basis of (a) mode of application and (b) chemical constitution. Classification of dyes based on chemical constitution is listed in **(Table 1.9)**.

Table 1.9: Classification of dyes based on their chemical constitution

S. No.	Class of Dyes	Examples	Remarks
1.	Nitroso	Fast green O, napthol green Y	Characterised by the presence of nitro group as chromophore and phenolic group as auxochrome in the o-position with respect to each other.
2.	Nitro	Martius yellow, napthol yellow - S	Characterised by the presence of – NO_2 group as chromophore.
3.	Anthraquinone	Alizarin red S, Alizarin blue	Characterised by the presence of chromophores =C=O and =C=C arranged in the anthraquinone complex.
4.	Diphenylmethane	Auramine O	Characterised by the presence of NH=C= group as chromophore. They also contain a diphenylmethane nucleus.
5.	Triphenylmethane	Malachite green, methyl violet	Characterised by the presence of quinonoid group, as their chromophore and $-NH_2$, NHR, $-NR_2$ (basic) or –OH (acidic) group as the auxochrome.
6.	Phthaleins	Phenolphthalein	These dyes are regarded as the derivatives of triphenyl methane.
7.	Xanthene	Eosin, Fluorescein	Characterised by the presence of =C=O= or =C=N– as chromophore.
8.	Thiazole	Primuline	Characterised by the presence of chromophores > C = N– as well as S–C ≡ etc.

9.	Azo	-	All azo dyes are chracterised by the presence of azo group (–N = N–).
	(i) Acid azo	Methyl orange, methyl red	Characterised by the presence of one or more acidic groups – SO_3H, –COOH and –OH.
	(ii) Basic azo	Aniline yellow, bismark brown	characterised by the presence of amino or substituted amino groups $–NH_2$, –NHR or $–NR_2$ as auxochrome.
	(iii) Mordant azo	Carmoisine, diamond black F	These dyes form complexes with mordants such as oxides of cromium, cobalt, copper, aluminium etc.
	(iv) Stilbene	Direct yellow R, direct yellow II	Characterised by the presence of –N=N– and =C=C= chromophores.
	(v) Ingrain or developed azo	Para red, naphthol – AS	

1.16 REVIEW OF LITERATURE

The work published on TLC of amino acids, vitamins, carbohydrates and nucleobases during the last fifteen years has been presented briefly in **Table 1.8-1.11** respectively.

Table 1.10: Literature appeared during 1995 – 2009 on chromatography of amino acids.

Title	Remarks	Ref.
Free amino acids in plant extracts by Thin layer chromatographic separation and identification of free amino acids.	Identification of free amino acids in extracts of medicinal plants on cellulose by TLC.	[55]
Kinetic detection of overlapped amino acids in thin-layer chromatography with a direct trilinear decomposition method.	Kinetic fluorescence detection of glycine and glutamine after TLC separation.	[56]
Simultaneous separation of amino acids, organic acids and nucleosides in clinical chemistry by two-dimensional thin-layer chromatography.	Simultaneous separation of amino acids, organic acids and nucleosides in clinical chemistry by two dimensional thin layer chromatography.	[57]
A comparative study of HPLC and TLC separation of amino acids using Cu (II) ion.	Utilization of Cu ion in TLC separation of amino acids with acetate buffer-acetonitrile-n-butanol mobile phase and the comparison of TLC results with by RP-HPLC.	[58]
Planar chromatographic direct separation of some aromatic amino acids and aromatic amino alcohols into enantiomers using cyclodextrin mobile phase additives.	Qualitative analysis of aromatic amino acids and aromatic amino alcohols on cellulose with highly concentrated solutions of α- or β- cyclodextrin by planar chromatography.	[59]

Title	Remarks	Ref.
Quantitative thin-layer chromatography of industrial amino acids.	Quantitative TLC of industrial amino acids employing video densitometric analytical technique.	[60]
Modified programmed multiple gradient development (MGD) in the analysis of complex plant extracts.	Separation of derivatives of amino acids on HPTLC silica layers following multiple gradient development (MGD) technique with different concentrations of ethyl acetate in heptane and chloroform.	[61]
Study on the limit test of other amino acid in amino acids by thin layer chromatography.	A rapid and reproducible TLC method for detection of amino acids on silica gel G with n-butanol- glacial acetic acid- water (3+1+1).	[62]
The separation of DL-enantiomers of amino acids with thin layer chromatography modified by α-cyclodextrin.	TLC separation of DL-enantiomers of amino acids on β- CD modified silica with urea and dicarboxylic acid containing eluent.	[63]
Derivative spectroscopy in conjunction with thin layer chromatography applied for determination.	Application of TLC in combination of derivative spectroscopy for determination of amino acids in baby foods on silica gel plates with n-butanol-acetic acid-water (4+1+1) and C_2H_2OH-water (70+3).	[64]
Separation of optical isomers of amino acids on modified chitin and chitosan layers.	Qualitative analysis of amino acids by TLC on chitin, chitosan, Cu impregnated chitin with three component mobile phase.	[65]

Title	Remarks	Ref.
TLC resolution of enantiomers of amino acids and dansyl derivatives using (1R, 3R, 5R)-2-azobicyclo [3, 3, 0] octan-3-carboxylic acid as impregnating reagent.	Resolution of enantiomers of amino acids and their dansyl derivatives by TLC using (1R, 3R, 5R)-2-azobicyclo[3,3,0] octan-3- carboxylic acid as impregnating reagent with mixtures of 0.5M aqueous NaCl and MeCN as mobile phase.	[66]
Thin-layer chromatographic separation of amino acid enantiomers using ligand exchange.	Qualitative analysis of amino acid enantiomers by TLC on silica gel treated with L-arginine and Cu acetate.	[67]
Topological indexes for evaluation of the separation of D and L amino acids by TLC.	TLC separation of amino acid enantiomers on chiral plates. Distinction between L and D isomers on the basis of the proposed topological indexes.	[68]
Quantitative analysis of L-lysine L-threonine, L-homoserine and cobalamines in fermentation broth.	Quantitave analysis of amino acids in fermentation broth on sorbfil TLC plates with mixed-aqueous solvents containing NH_3.	[69]
Quantitative analysis of L-tryptophan in fermentation broth.	Quantitave analysis of L-Tryptophan on sorbfil TLC plates with propan-2-ol-25% aqueous NH_3.	[70]
Micellar thin layer chromatographic separation and identification of amino acids: Separation of L-proline from some aliphatic and aromatic amino acids.	Separation of L-proline from other aliphatic and aromatic amino acids by micellar TLC on plain alumina.	[71]

Title	Remarks	Ref.
Detection, separation and analysis of α-amino acids by means of TLC using 4 dipropylamino-diazabenzene-4'iso- thiocyanate (DPABITC).	Detection, separation and analysis of α-amino acids by means of TLC using 4-dipropylamino-diazabenzene-4-iso-thiocyanate (DPABITC).	[72]
Application of microemulsions in thin layer chromatographic analysis of amino acids.	TLC analysis of amino acids using microemulsion systems as mobile phase on silica gel.	[73]
Resolution of enantiomers of DL-amino acids on silica gel plates impregnated with optically pure (-)- quinine.	TLC resolution of enantiomers of racemic amino acids was achieved on silica gel plates impregnated with optically pure (-)-quinine using butanol-chloroform-acetic acid and Et acetate-CCl_4-propionic acid systems.	[74]
Separation of amino acids on alumina layer developed with oil-in-water microemulsion.	TLC separation of aliphatic and aromatic amino acids on plain alumina and impregnated alumina with o/w microemulsion.	[75]
Effect of mobile phase composition and pH on thin layer chromatographic behaviour of amino acids.	Thin layer chromartographic behaviour of amino acids was studied on plain silica gel 'G' layers in one component, two-component and three-component mobile phases in varying ratios.	[76]
Thin layer chromatography of amino acids on titanium tungstate using dimethyl sulfoxide as the mobile phase.	Dimethyl sulfoxide was used as the mobile phase for TLC of amino acids on titanium tungstate.	[77]

Title	Remarks	Ref.
Identification of amino acids on alumina TLC plates developed with aqueous anionic surfactant solutions containing inorganic and organic additives: selective separation of L-proline from other amino acids.	TLC performed on alumina layer and 0.01 M aq. SDS plus 0.1M aq. $CuSO_4$ in volume ratio (9: 1) as mobile phase.	[78]
Application of a new developer in thin layer chromatographic analysis of amino acids.	The chromatographic behavior of amino acids on the silica gel thin layers using CTAB-Bu alc-n-octane-water microemulsion as a developer was studied.	[79]
Simple methodology for the purification of amino acids.	The isolation and separation of amino acids was carried out by silica gel thin layer chromatography using as a eluent a mixture of isopropanol-methanol-NH_3 (0:1:1) to (9:1:0.5).	[80]
Two new spray reagents for detection of amino acids on thin-layer plates.	The proposed reagents (4-hydroxyacetophenone-isatin and 4-hydroxybenzaldehyde-isatin) were used for the formation of distinguishable colors with amino acids on TLC plates.	[81]
Detection of proline, arginine, and lysine using iodine-azide reaction in TLC and HPTLC.	Application of a modified I-azide procedure for detection of proline, arginine and lysine by means of TLC and HPTLC is described. The developed plates were sprayed with a mixture of Na azide and starch solution, adjusted to pH 5.5, and exposed to I_2 vapor.	[82]

Title	Remarks	Ref.
Selective TLC separation of lysine and threonine in pharmaceutical preparations.	TLC of α-amino acids has been performed on layers prepared from a 1: 4 stannic arsenate-cellulose mixture. Lysine and threonine were selectively separated and quantitatively determined from among the mixture of amino acids present in a common available drug.	[83]
Study on a quantitative method for arginine derivatives in ginseng products after processing.	TLC performed on silica gel H plates, developed by n-butanol-acetic acid-water (4:1:5).	[84]
Reversed phase thin layer chromatography of amino acids on silicone fluid DC 200, triaryl phosphate and tri-n-butylamine impregnated silica gel-G layers.	RP-TLC on silicone fluid DC 200, triaryl phosphate (TAP) and tri-n-butylamine (TBA) impregnated silica gel-G layers in two component mobile phases (n-propanol-water).	[85]
Amino acids in field violet and tall plaster clover.	Analysis of amino acids in the field violet and tall plaster clover by TLC and amino acid analyzer.	[86]
Chromogenic charge transfer cleft-type tetrahydrobenzoxanthene enantioselective receptors for dinitrobenzoylamino acids.	Racemic mixtures of the receptor can be resolved with TLC impregnated with the guest and structure of the complexes was studied by X-ray.	[87]

Title	Remarks	Ref.
A new spray reagent for identification of amino acids on thin-layer chromatography plates.	A new spray reagent (2, 3-dichloro-1, 4-napthoquinone) with high sensitivity identify amino acids on TLC plates.	[88]
TLC analysis of free amino acids in valerianae radix and its products.	TLC performed on cellulose and silica gel using two solvent systems. N-butanol-acetone-glacial acetic acid- water (35: 35: 10: 20 by vol) and Et. Acetate-methanol-citric acid/phosphate buffer (pH = 6) (30: 30: 40 by vol).	[89]
A new reagent for identification of amino acids on thin-layer chromatography plates.	4-Hydroxyacetophenone/isatin-5-sulfonic acid (sodium salt), for identification of amino acids on TLC plates.	[90]
Effect of pH and salts on the binding of free amino acids to the corn protein zein studied by thin-layer chromatography.	The interaction of free amino acids with the corn protein zein was studied by TLC on cellulose layers covered with zein and the effect of pH and salts on the strength of interaction was elucidated.	[91]
Separation of amino acids as phenyl thiocarbamyl derivatives by normal and reversed phase thin-layer chromatography.	Separation of amino acids on normal-phase and reversed-phase TLC using sodium azide, starch and iodine vapor.	[92]
Quantitative determination of gamma-aminobutyric acid in nutritional supplement products by high-performance thin-layer chromatography.	HP-TLC silica gel layer, ninhydrin spray reagent, automated sample application densitometric scanning for quantitative determination of gamma aminobutryic acid.	[93]

Title	Remarks	Ref.
Mobility behaviour of amino acids through soil TLC.	Transportation of amino acids through static flat bed of buffered soil (pH 2.06) as stationary phase and w/o micro-emulsion as eluent.	[94]
Role of sulfur compounds in the detection of amino acids by ninhydrin on TLC plate.	Three new sulfur reagents for specific identification of amino acids on TLC plates.	[95]
TLC separation of L-Tryptophan using microemulsion mobile phase and its spectrophotometric determination.	Selective separation of L-tryptophan from other amino acids by TLC on silica gel with w/o microemulsion.	[96]
A new TLC system for specific separation of L-Arginine from other non-essential amino acids.	TLC on SDS impregnated silica gel and borate phosphate buffer was performed for specific separation of L-Arg from L-Ser and L-Tyr.	[97]
New reagent for detection of amino acids on TLC plates.	2, 3-dichloro-1,4-naphthaquinone and isatin were introduced as a new reagent for the detection of amino acids on silica gel TLC plates with very high sensitivity.	[98]
Multiple development HPTLC analysis of amino acids on cellulose layers.	Multiple development technique (UMD and IMD) were used for studying retention behavior of amino acids on cellulose layers.	[99]
Effect of mobile phase pH on chromatographic behavior in chiral ligand-exchange thin-layer chromatography (CLETLC) of amino acid enantiomers.	Amino acids enantiomers were separated on commercial chiral TLC plates in reversed phase mode.	[100]

Title	Remarks	Ref.
Surfactants modified silica static phase for sorption studies of essential amino acids by thin layer chromatography.	TLC performed on SDS impregnated silica layer and borate-phosphate buffer of pH 2.3 to separate L-His from DL-Trp.	[101]
Mixed surfactants enable separation of lysine from other essential amino acids in TLC on silica gel.	TLC of eight essential amino acids performed on silica layers with Triton X-100-sodium dodecyl sulfate- acetone (1:1:5) v/v.	[102]
Thin layer chromatographic analysis of amino acids on high performance silica TLC plates with buffered aqueous eluents: Separation of DL-phenylalanine from L- tyrosine.	Silica gel $60F_{254}$ HPTLC plates and buffered $ZnSO_4$ solution (pH 2.3) used for mutual separation of DL-Phe and L-Tyr.	[103]
Separation of coexisting tryptophan, alanine and phenyalanine or tyrosine by silver ion high-performance thin-layer chromatography.	Ag ion impregnated silica gel HPTLC plates and borate buffer as mobile phase was used for the separation of amino acids.	[104]
Mobility behavior of amino acids on silica static phase : Micelles activated separations.	Separation of amino acids by TLC using mobile phases containing mixed micelles as well as 1-butanol and aprotic organic solvents as modifying additives has been used.	[105]
Chiral ligand exchange chromatography of amino acids using porous graphitic carbon coated with a dinapthyl derivative of neamine.	Preparation and the evaluation of porous graphitic carbon column coated with a new dinaphthyl derivative of neamine for chiral ligand-exchange of amino acids.	[106]

Title	Remarks	Ref.
Chromatographic separation of enantiomers of non-protein alpha amino acids after derivatization with Marfey's reagent and it's four variants.	Some non protein alpha amino acids were derivative with Marfey's reagent and its four variants and the resultant diastereomers were separated by normal and reversed phase TLC and RP-HPLC.	[107]
Thin Layer chromatographic analysis of free amino acids in haemolymph and DGG of Indoplanorbis exustus (Mollusca: Gastropoda) naturally infected with Digenetic trematodes.	A preliminary TLC, followed by HPTLC analysis was used to determine the qualitative status of free amino acids in the uninfected and infected snails.	[108]
Use of Carbohydrates as Eluent in Thin Layer Chromatographic Separation of Amino acids on Conventional Stationary Phases.	Thin layer chromatographic studies of amino acids were performed on three differentially charged surfaces of silica gel, alumina and cellulose with 40% aqueous solution of five carbohydrates namely dextrose, fructose, maltose, lactose and sucrose.	[109]

Table 1.11: Literature appeared during 1995 – 2009 on chromatography of vitamins.

Title	Remarks	Ref.
Quantitative determination of vitamin B complex constituents by fluorescence quenching after TLC separation	Cellulose and newly synthesized carbamide, formaldehyde 1 polymer aminoplast TLC plates were developed on specific mobile phase 1-butanol-water-acetone (25:9:5 v/v/v) and 1-butanol-methanol-benzene water (20:10:10:8 v/v/v/v)	[110]
RPTLC and derivative spectrophotometry for the analysis of selected vitamins	RP-18 plates were used and methanol, ethanol, benzene, chloroform were used as eluent in different proportions	[111]
TLC separation of isomers of ascorbic acid and dehydro ascorbic acid	Sodium borate (RP-B) TLC plates with and without meta phosphoric acid were used	[112]
Mineral and water soluble vitamin content in the Kombucha drink	Silica gel G plates were developed with water as mobile phase and extraction of vitamin B_1 (thiamine) from Kombucha drink. AAS also used for determination of vitamins	[113]
Second derivative UV spectrophotometry and HPTLC for the simultaneous determination of vitamin C and dipyrone	Water + MeOH (95:5) an silica gel plates were used and vitamin C was determined on pharmaceutical tablets	[114]
Separation of vitamin B complex and folic acid by HPLC, normal phase and reversed phase TLC	The stationary phase RP-18 plates and acetonitrile-borate buffer and acetonitrile methanol as mobile phase were used	[115]

Title	Remarks	Ref.
Development and application of new C_{30}–modified TLC plates.	The C_{30} plates were prepared by the modification of silica gel plates with triacontyltrichlorosilane, using different ratios of the modifying agent.	[116]
Comparison of methods by TLC and HPTLC for determination of aflatoxin M_1 in milk and B_1 in eggs.	Comparison of TLC and HPTLC. Determination of aflatoxin M_1 in milk and B_1 in eggs.	[117]
Research of xanthinol nicotine quality by thin layer chromatography.	Quality determination of xanthinol nictotine by TLC.	[118]
Comparison of chromatographic separation of selected nicotinic acid derivatives by TLC and HPLC techniques.	Separation of nicotinic acid and its derivatives analyzed by adsorption and reversed phase TLC and HPLC were compared.	[119]
Development and validation of a method for quantitative determination of pyridoxine hydrochloride (vit. B_6) in nutritional supplements using high performance thin layer chromatography with densitometric scanning of fluorescence- quenched zones.	Development on silica gel HPTLC plates with chloroform ethanol-acetone- ammonia (2:2:2:1) mobile phase, detection as a fluorescence-quenched zone at 254 nm, and quantification using scanning densitometry.	[120]

Title	Remarks	Ref.
Chromatographic study of photolysis of aqueous cyanocobalamin solution in presence of vitamin B and C.	Chromatographic study of photolysis of cyanocobalamin.	[121]
Determination of tocopherol concentration in certain autochthonous cereals.	Tocopherols in wheat, barley and oat were detd. in solvent exts. by spectrophotometry and isomers were separated by TLC.	[122]
Thin-layer chromatographic analysis of hydrophilic vitamins in standards and from Helisoma trivolvis snails.	Separation of hydrophilic vitamins using 14 mobile phases, precoated silica gel and chem. bonded silica gel TLC and high performance TLC plates. Best sepn. on silica gel plates with 1-butanol-chloroform-acetic acids-ammonia-water, 7:4:5:1: 1, benzene- methanol- acetone-acetic acid, 70:20:5:5, and chloroform-ethanol- acetone ammonia, 2:2:2:1 as mobile.	[123]
Study of lipophilicity and application of selected structural descriptors in QSAR analysis of nicotinic acid derivatives. Investigation on RP18WF$_{254}$ plates : Part II.	Nicotinic acid and its derivatives were investigated on RP18WF$_{254}$ HPTLC plates with methanol-water in different volume proportions as mobile phases.	[124]
Determination of L-ascorbic acid after chromatographic separation.	TLC separation of L-ascorbic acid and detected by UV at λ = 254 nm. Determination of L-ascorbic acid in complex pharmaceuticals and in pepper juice samples.	[125]

Title	Remarks	Ref.
Quantitative determination of α-tocopherol in Pistacia lentiscus, Pistacia lentiscus var. chia and pistacia terebinthus by TLC-densitometry and colorimetry.	A quantitative determination of α-tocopherol by TLC-densitometry and colorimetry.	[126]
Hydrophilic vitamins TLC separation and the possibility of their quantitative determination.	Separation of the hydrophilic vitamins by thin layer chromatography (TLC), using fluorescent silica get plates as a stationary phase and two mobile phases were used.	[127]
Chromatographic control of biological activity of fat soluble vitamins (A, D, E) in food and feed production.	Determination of fat soluble vitamins (A, D, E) by TLC in food and feed products on silufol UV-254- TLC plates using hexane / acetone solvent mixture (4:1).	[128]
Qualitative and semiquantitative TLC analysis of vitamins of vitamine A, D and E.	Determination of vitamins A,D_2 and E from mixtures by $60F_{254}$ TLC plates on plastic sheets and n-hexane / ether (9:1) and benzene/chloroform (1:1) as mobile phases for vitamins A and D_2 resp. D_2 and E.	[129]
Determination of niacinamide in vitamins with minerals tablets by TLC.	Developer was chloroform-ethanol-water (54:27:5). Detection wavelength was 262 nm and ref. wavelength was 320 nm.	[130]
Simultaneous determination of water- and fat-soluble vitamins by high-performance thin layer chromatography using an aqueous micellar mobile phase.	Fat soluble vitamins A and E and water soluble vitamins B (B_1, B_2, B_6 and B_{12}) can be separated by HPTLC using fractional elution. Benzene was used as the first mobile phase and a 0.02 M aq. micellar solution of SDS was the second eluent.	[131]
Application of densitometry for the evaluation of the separation effect of nicotinic acid derivatives. Part III. Nictonic acid and its derivatives.	Nicotinic acid and its derivatives were studied by NP-TLC and RP-HPTLC.	[132]

Title	Remarks	Ref.
Identification and simultaneous separation of six hydrophilic therapeutic vitamins by micellar thin layer chromatography.	4% aq SDS + acetonitrile (1:2, v/v) and silica gel 60 F_{254} HPTLC plates to resolve multecomponents mixture of vitamins. Visualized under UV radiation (1 = 254).	[133]
Influence of impregnation of a mixture of silica gel and kieselguhr with copper (II) sulphate (VI) on profile change of the spectrodensitograms and the R_F values of nicotinic acid and its derivatives.	Nicotinic acid and its derivatives were separated using adsorption TLC on aluminum plates precoated with a mixture of silica gel 60 and kieselguhr $F_{254,}$ non impregnated and impregnated with 2.5% and 5% aq. solutions of $CuSO_4$. Acetone-n-hexane in different volume compns. Was used as mobile phase.	[134]
Micelles activated planar chromatographic separation of hydrophilic vitamins.	Mixed micelles (aq solution of anionic SDS (0.5%) + nonionic triton X 100 (0.5%) surfactants) and butanol with silica gel was used. The method is applicable to the identification and separation of vitamins (B_1, B_9 and C) present in drug samples (Becasule and Celin).	[135]
Evaluation of the lipophilicity of fat-soluble vitamins.	Ergocalciferol, cholecalciferol, (±)-α tocopherol, tocopherol acetate, retinol, retinol acetate, retinol palmitate, menadione, and phytonadione have been investigated on $RP8F_{254S}$ and $RP18F_{254S}$ TLC plates with methanol-water in different volume proportions as mobile phases.	[136]
Simultaneous separation and identification of cyanocobalamin,thiamine and ascorbic acid on polyoxyethylene sorbitan monooleate-impregnated silica layers with water as mobile phase.	TLC system comprising a silica layer impregnated with nonionic surfactant (2% cween 80) as stationary phase and double distilled water as mobile phase were used.	[137]

Table 1.12 : Table 1.11: Literature appeared during 1995 – 2009 on chromatography of carbohydrates .

Title	Remark	Ref.
Thin layer chromatographic and hydrolysis methods for the identification of plant gums in art object.	The TLC system evaluated precoated silica gel + 1-propanol: water: ammonium hydroxide (79:20:1) with p-inisidine phthalate as a reaction agent for the resolution of all the eight possible sugars of gums.	[138]
Determination of carbohydrates in soluble coffee by anion-exchange chromatography with pulsed amperometric detection: interlaboratory study.	Carbohydrates were separated on a pellicular anion-exchange column using pure water as mobile phase and were detected by pulsed amperometry.	[139]
High-performance anion-exchange chromatography of carbohydrates using a new resin and pulsed amperometric detection.	Study on the use of a new polymer-based strong anion-exchange stationary phase for rapid and selective separation of carbohydrates and related compounds by high-pH anion-exchange chromatography with pulsed amperometric detection.	[140]
Copper electrodes for stable subpicomole detection of carbohydrates in high-performance liquid chromatography.	A new pretreatment of copper (Cu) electrodes used for catalytic oxidation of carbohydrates is presented. Glucose was detected at concentration levels as low as 0.2 pmole with a dynamic range up to 10 000 p mole over a period of 1 month.	[141]
Development of protamine-bonded phase for separation of saccharides in liquid chromatography.	A protamine-bonded polymer gel was synthesized for versatile separation of monosaccharides, oligosaccharides, sugar alcohols and uronic acids in liquid chromatography. This column was used for the analysis of saccharides in urine using a post-column reaction detection system.	[142]

Title	Remark	Ref.
Recent progress in carbohydrate separation by high-performance liquid chromatography based on size exclusion.	Developments in detection systems for high-performance SEC for the determination of molecular-size and molecular-weight distributions of polymers on a sound theoretical basis and increased the range of information on molecular characteristics that can be retrieved from SEC data.	[143]
Determination of fructose, glucose and sucrose in beverages by high-performance thin layer chromatography.	A method is described for the simultaneous determination of fructose, glucose and sucrose in beverages such as sodas and iced teas by thin layer chromatography of samples and standards on channeled preadsorbent high performance silica gel plates and densitometric scanning.	[144]
Automated multiple development HPTLC analysis of sugars on hydrophilic layers: II. Diol layers.	After the systematic study of the analysis of sugars by AMD on hydrophilic layers, results are presented on diol layers which seem to be very selective and suitable for practical applications.	[145]
Detection of carbohydrates by electrospray ionization-ion mobility spectrometry following microbore high-performance liquid chromatography.	This paper reports the first example of electrospray ion mobility spectrometry as a detection method for HPLC separation. Reduced mobility constants (K_0) for 21 carbohydrates, including simple sugars, sugar alcohols and amino sugars, were determined to range from 0.68 to 1.37 $cm^2 V^{-1} s^{-1}$.	[146]

Title	Remark	Ref.
High-performance anion-exchange chromatographic separations of carbohydrates on a macrocycle-based stationary phase with eluents of relatively low pH and concentration.	A high-pH anion-exchange chromatography stationary phase was prepared by adsorbing cryptand *n*-decyl-[2.2.2] (D222) onto polymeric resins. Alditols, and mono- and disaccharides were separated isocratically with 13 mM KOH.	[147]
Determination of blood glucose by quantitative TLC on preadsorbent silica gel plates.	A TLC method was developed for quantification of glucose in human blood serum using preadsorbent silica gel plates, acetonitrile-water (85:15) mobile phase, methanolic sulfuric acid detection reagent and scanning densitometry.	[148]
On-line monitoring of monosaccharides and ethanol during a fermentation by microdialysis sampling, liquid chromatography and two amperometric biosensors.	Two amperometric biosensors were used as detection units in liquid chromatography for on-line monitoring of the production of fuel ethanol during fermentation.	[149]
TLC and GC–MS identification of glucose and maltose in *Biomphalaria glabrata* (gastropoda), and use of quantitative TLC to determine the effect of starvation on the amounts of these carbohydrates.	Maltose and glucose have been identified as the primary carbohydrates in the planorbid snail *Biomphalaria glabrata* by HPTLC on silica gel, amino-bonded, C_{18} reversed-phase, and cellulose plates.	[150]
High-performance thin-layer chromatographic analysis of sugars in snail-conditioned water and mucus from *Biomphalaria glabrata*, *Helisoma trivolvis*, and *Lymnaea elodes*.	The presence of sugars, particularly glucose, maltose and sucrose in the snail-conditioned water of *Biomphalaria glabrata*, the two strains of *Helisoma trivolvis*, and *Lymnaea elodes* was variable, although only maltose was quantifiable in two samples of snail conditioned waters from *B. glabrata*.	[151]

Title	Remark	Ref.
Determination of maltodextrins in enteral formulations by three different chromatographic methods.	Maltodextrins (G_1 to G_{11}) present in enteral formulations have been determined by three analytical methods — TLC, high-performance anion-exchange chromatography with pulsed electrochemical detection (HPAEC-PED) and high-performance liquid chromatography with refractive-index detection (HPLC-RI).	[152]
Optimization of the capillary gas chromatographic analysis of mono- and oligosaccharides in honeys.	A capillary gas chromatographic method has been developed for simultaneous determination of at least 18 sugars (2 monosaccharides, 11 disaccharides and 5 trisaccharides) in honey as their oxime-trimethylsilyl ethers.	[153]
Determination of resistant short-chain carbohydrates (non-digestible oligosaccharides) using gas–liquid chromatography.	A technique for the measurement of resistant SCC (RSCC), which are not susceptible to pancreatic amylase or the brush border enzymes.	[154]
Impregnation of silica gel with inorganic salts from the standpoint of specific adsorption theory. The effect of this impregnation on the separation of sugars by TLC.	Results from the separation of some neutral sugars on silica gel impregnated with sulfates, chlorides and nitrates of various metals are presented. The effect of pH and of the conc. of the impregnating solution was studied.	[155]
Determination of sugar alcohols in confectioneries by high-performance liquid chromatography after nitrobenzoylation.	A method was developed for the determination of sugar alcohols, *meso*-erythritol, xylitol, D-glucitol, D-mannitol, maltitol and parachinit by high-performance liquid chromatography (HPLC). HPLC was performed on a phenyl column, using acetonitrile–water (67:33) as mobile phase and UV detection (260 nm).	[156]

Title	Remark	Ref.
Determination of carbohydrates in food samples by capillary electrophoresis with indirect UV detection.	Under optimized conditions, 28 carbohydrates including mono- and disaccharides, uronic acids, sialic acids, amino sugars and sugar alcohols were separated in less than 25 min. The detection limits for fructose, glucose and sucrose were in the range from 12 to 16 mg l^{-1} with pressure injection of 50 mbar for 6 s (ca. 6 nl) at a signal to noise ratio of 3.	[157]
TLC separation of some common sugars on silica gel plates impregnated with transition metal ions.	TLC separation of glucose, maltose, lactose, sorbitol and sucrose on silica gel plates impregnated with transition metal ions Cu (II), Ni (II), Zn (II) or Cd (II) was achieved.	[158]
Separation and determination of alditols and sugars by high-pH anion-exchange chromatography with pulsed amperometric detection.	Carbohydrates such as alditols (polyols or sugar alcohols), monosaccharides and disaccharides are separated as anions by anion-exchange chromatography with a sodium hydroxide eluent. It determines all the polyols used as food additives in food products and the most commonly found mono- and disaccharides on a routine basis.	[159]
Automated flow system on-line to LC with postcolumn derivatization for determination of sugars in carbohydrate-rich foods.	A method for the direct determination of carbohydrates in foods using an automated-flow system coupled on-line to a high-performance liquid chromatograph is proposed.	[160]
Determining sugar composition of food gum polysaccharides by HPTLC.	Sugars and other gums were determined by comparison of R_F values with standards by HPTLC on Si 50000 plates after acid hydrolysis of the polysaccharides.	[161]

Title	Remark	Ref.
Analysis of monosaccharides in bovine, caprine and ovine κ-casein macropeptide by gas chromatography.	Monosaccharides present in 1c-casein macropeptide (CMP) from bovine, ovine and caprine milks have been determined by gas chromatography after acid hydrolysis with trifluoroacetic acid (TFA) or methanolysis with HCI-methanol.	[162]
Differences in sialic acid density in pathogenic and non-pathogenic *Aspergillus* species.	Mild acid hydrolysis and purification of conidial wall carbohydrates yielded a product that had the same R_F as the Neu5Ac standard when analysed by high-performance thin-layer chromatography.	[163]
Detection of oligosaccharides in sugar products using planar chromatography.	A simple and reliable analytical technique to be used for the detection of oligosaccharides in different materials.	[164]
GC-MS analysis of monosaccharide mixtures as their diethyldithioacetal derivatives: Application to plant gums used in art works.	A simple GC-MS method has been studied for the determination of aldoses and uronic acids in polysaccharide hydrolysates. The obtained diethyldithioacetal trimethylsilylates or peracetates are separated by capillary column gas chromatography on SE-54 stationary phase.	[165]
TLC separation of carbohydrates on silica gel modified with copper (II) salts.	Impregnation of silica gel was conducted by immersion method with the help of copper (II) sulfate and ammonia complex: $Cu(NH_3)_4(2+)$. [please check it] The adsorbents thus obtained were used for the analysis of carbohydrates of mono-di-oligosaccharides.	[166]
The effect of metal salts on the separation of carbohydrates by TLC.	Carbohydrates have been examined by TLC on layer modifies with metal ions, in the form of salts, by dipping commercial or methanolic solu. or by use of mobile phase additives, the mobile phases were mix. of acetoniltrile, propan-2-ol, or acetone with water or aq. solu. of the metal salts.	[167]

Title	Remark	Ref.
Chromatographic and spectroscopic analysis of the fluorescent compounds derived from monosaccharides on HPTLC-NH_2 plates.	In this paper, results were presented from recent studies focusing on elucidation of the mechanism of visualization of simple sugars (e.g. D-(+)-glucose, D-(+)-galactose, and D-(–)-fructose) developed on glass.	[168]
High-performance liquid chromatographic separation of carbohydrates on a stationary phase prepared from polystyrene-based resin and novel amines.	HPLC separations of monosaccharides (sorbitol, fucose, glucosamine, mannose, glucose, galactose, fructose, allose and altrose) and disaccharides (trehalose, lactose, cellobiose and maltose) were performed successfully on new anion-exchange stationary phases O_n (n=1, 2 and 3) with a dimethylamino terminal functional group.	[169]
Precolumn derivatization of reducing carbohydrates with 4-(3-Methyl-5-oxo-2-pyrazolin-1-yl) benzoic acid. Study of reaction, high-performance liquid chromatographic separation and quantitative performance of method.	A simple method for quantitative determination of carbohydrates by reversed-phase, high-performance liquid chromatography after pre-column derivatization and UV detection has been developed.	[170]
Determination of 1-phenyl-3-methyl-5-pyrazolone-labeled carbohydrates by liquid chromatography and micellar electrokinetic chromatography.	Five monosaccharide derivatives have been well separated by MEKC and HPLC under optimized conditions. The developed methods have been successfully applied to the analysis of carbohydrates in aloe powder and food.	[171]
Simultaneous analyses of neutral carbohydrates and amino sugars in freshwaters with HPLC–PAD.	Determination of concentrations of neutral and amino sugars and a sugar alcohol in freshwaters using high-performance liquid chromatography and pulsed amperometric detection with a single isocratic analysis. Coeluting arabinose, galactosamine, and mannosamine are separated with a mobile phase of 22.8mM NaOH-KOH at a temperature of 17°C.	[172]

Title	Remark	Ref.
TLC determination of mannitol and lactulose on amino HPTLC plates.	An analytical method has been developed for determination of lactulose and mannitol in urine on the same amino HPTLC plate.	[173]
Fingerprint profiling of acid hydrolyzates of polysaccharides extracted from the fruiting bodies and spores of Lingzhi by high-performance thin-layer chromatography.	Modern extraction and planar chromatographic instrumentation were employed for the fingerprint profiling of carbohydrates from an important and popular medicinal mushroom commonly known as Lingzhi.	[174]
The possibility of TLC-FID detection in oligosaccharide analysis.	Chromatography was performed on Chromarods S III with two different mobile phases, ethyl acetate – formic acid – water and butanol – ethanol – water. Pretreatment of the biological samples was minimal.	[175]
Preliminary TLC analysis of fructooligosaccharides as feed additives.	A rapid, simple qualitative thin-layer chromatographic method has been developed for separation of mono- and fructooligosaccharides from feed additives with the aim of analyzing them in biological materials.	[176]
Study and application of developing agents in thin layer chromatography for honey sugars.	A TLC study for honey sugar was established in which the mobile phase was glacial acetic acid- chloroform- 95% ethanol (21:23:15v/v/v) and the stationary phase was 0.2 mol/L sodium acetate- silica gel G.	[177]

Title	Remark	Ref.
Analysis of low molecular weight carbohydrates in food and beverages: A review.	This review presents a study of new advances in chromatographic methods and capillary electrophoresis for the analysis and quantification of carbohydrates in food and drink.	[178]
Application of curve fitting in thin-layer chromatography–flame ionization detection analysis of the carbohydrate fraction in marine mucilage and marine snow samples from italian seas.	The joint- approach TLC–FID analysis and deconvolution procedure allows for the characterization of the carbohydrate fraction of the marine samples in a single step without using the different derivatization procedures requested by the most common gas chromatography and high-performance liquid chromatographic methods for carbohydrate analysis.	[179]
Analysis of carbohydrates in beverages by capillary electrophoresis with precolumn derivatization and UV detection.	A simple, selective capillary electrophoretic method using precolumn derivatization and UV detection at 280 nm to determine carbohydrates in beverages. Three carbohydrates, glucose, maltose and maltotriose, were analysed. The labelling reagent was *p*-aminobenzoic acid (PABA).	[180]
Routine method for quantification of starch by planar chromatography (HPTLC).	The starch was hydrolyzed using α-amylase and amyloglucosidase and the resulting glucose was separated on silica gel 60 HPTLC plates and quantified at λ = 520 nm after derivatization.	[181]

Title	Remark	Ref.
Analysis of carbohydrates in plants by high-performance anion-exchange chromatography coupled with electrospray mass spectrometry.	A mass spectrometer was coupled to high-performance anion-exchange chromatography (HPAEC) with the help of electrochemical neutralization of the eluent and post-column addition of lithium chloride for carbohydrate analysis. Parallel selective channels (single ion monitoring) were used to decrease the detection limits and separate unresolved peaks.	[182]
Subcritical water as eluent for chromatographic separation of carbohydrates using cation-exchange resins.	The separation of sugars and sugar alcohols with a strong cation-exchange poly(styrene-co-divinylbenzene) (PS-DVB) resin in the Na^+ form using subcritical (superheated) water as an eluent.	[183]
Separation of complex fructo-oligosaccharides (FOS) and inulin mixtures by HPTLC-AMD.	A first attempt at the separation of fructo-oligosaccharides (FOS) and inulin mixtures is presented. Preliminary results obtained by automated multiple development (AMD) of diol layers with an acetonitrile-acetone-water polarity gradient are reported.	[184]
High-performance anion exchange chromatography with pulsed amperometric detection for simultaneous determination of monosaccharides and uronic acids.	The chromatographic separation was carried out on a CarboPac PA20 anion-exchange column using NaOH and NaOAc (50–200 mM) as eluent at the flow rate of 0.5 ml min^{-1}. The separation was completed in 30 min. Detection limits of 2.5–14.4 μg l^{-1} (25 μl injection, 3 times of baseline noise) were achieved under the optimized conditions. The method had been applied for the determination of eight monosaccharides and two uronic acids in hydrolysates of polysaccharide and wood hemicellulose.	[185]

Title	Remark	Ref.
Analysis of amino acids and carbohydrates in green coffee.	During the course of the maillard reaction potentially harmful substances like acrylamide or 5-hydroxymethyl-furfural accrue as well. The carbohydrates were analysed by anion-exchange chromatography with pulsed amperometric detection and the amino acids by reversed phase chromatography after derivatization with 6-amino-quinolyl-*N*-hydroxysuccinimidyl carbamate and fluorescence detection.	[186]
Separation of *O*- and *C*-allyl glycoside anomeric mixtures by capillary electrophoresis and high-performance liquid chromatography.	In MEKC, the formation of chromophoric and charged complexes between the saccharides and borate as well as the hydrophobic interactions with micelles jointly contributed to the selective separation and sensitive detection of all the investigated anomeric couples.	[187]
Use of deconvolution methods for the analysis of sugars in kiwi juice by HPLC.	Three deconvolution algorithms were tested so that the concentration of sugars in samples of kiwi juice can be calculated accurately. The concentrations of glucose and fructose increase almost 150% at the end of six months of storage and increase nearly 131% for sucrose.	[188]
Determination of oligosaccharides by conventional high-resolution gas chromatography.	A simple gas chromatographic method for the analysis of (higher) oligosaccharides without needing a high-temperature procedure.	[189]
Rapid separation on activated charcoal of high oligosaccharides in honey.	A method using different proportions of water/ethanol solutions and activated charcoal was optimised to fractionate honey carbohydrates.	[190]

Title	Remark	Ref.
Analysis of sugars by micellar liquid chromatography with UV detection.	A micellar liquid chromatographic procedure is described for simultaneous determination of sugars previously derivatized with 4-amino-benzoic acid ethyl ester (4-ABEE).	[191]
Separation and quantitative determination of aldoses and alditols by over-pressured layer chromatography (OPLC).	A new OPLC method has been established for separation and quantitative determination of three alditols (D -xylitol, L -arabitol, and D -glucitol) and four aldoses (D -xylose, L -arabinose, D -glucose, and L -rhamnose).	[192]
Determination of saccharides in atmospheric aerosol using anion-exchange high-performance liquid chromatography and pulsed-amperometric detection.	Limits of detection for individual sugars are in the range 0.02–0.05 $\mu g\ mL^{-1}$ in solution, corresponding to 2–5 $ng\ m^{-3}$ from a 20 m^3 air sample. The overlap of arabitol and levogluocosan is overcome by using a Dionex PA-1 column, with appropriate control of eluent composition, and peak deconvolution software, allowing quantification of both sugars in difficult summer samples containing low-levels of levoglucosan.	[193]
Compositional monosaccharide analysis of transgenic corn glycoproteins by HPLC with fluorescence detection and LC–MS with sonic spray ionization.	A sensitive and reliable analytical method is developed to investigate protein for the presence of glycans by monitoring the monosaccharide composition. Identification and quantitation of low-level monosaccharides in the glycoprotein hydrolyzate are accomplished by derivatization prior to high-performance liquid chromatography (HPLC)-fluorescence and liquid chromatography (LC)-sonic spray ionization (SSI)-mass spectrometry (MS) analyses.	[194]

Title	Remark	Ref.
Direct detection method of oligosaccharides by high-performance liquid chromatography with charged aerosol detection.	A simple and rapid detection method of oligosaccharides using high-performance liquid chromatography with charged aerosol detection (HPLC-CAD) was studied. The direct detection of a sialylglycopeptide (SGP) derived from egg yolk was accomplished by HPLC-CAD using an amido-silica column, and its limit of detection was 0.40 pmol [signal-to-noise ratio (*S/N*) = 3].	[195]
Recent developments in sample preparation for chromatographic analysis of carbohydrates.	This review summarizes the most important treatments which have been recently developed to be applied prior to the analysis of carbohydrates by chromatographic techniques.	[196]
Separation and determination of carbohydrates in drinks by ion chromatography with a self-regenerating suppressor and an evaporative light-scattering detector.	Analysis of glucose and other carbohydrates are often performed by use of normal phase HPLC methods with acetonitrile as major eluent coupled with evaporative light-scattering detector (ELSD) or by use of anion-exchange ion chromatography (IC) methods with NaOH as eluent coupled with pulsed amperimetric electrochemical detector.	[197]
Resolution of oligosaccharides in glycopeptides using immobilized endo-M and ultra-performance liquid chromatography with electrospray ionization time-of-flight mass spectrometry.	The resolution of asparagine-linked oligosaccharides in glycopeptides was carried out by combination of the transglycosylation reaction and ultra-performance liquid chromatography with electrospray ionization time-of-flight mass spectrometry (UPLC-ESI-TOF-MS).	[198]

Title	Remark	Ref.
Determination of mannitol and sorbitol in infusion solutions by capillary zone electrophoresis using on-column complexation with borate and indirect spectrophotometric detection.	The separation was carried out in a fused silica capillary (total length 60 cm, effective length 50 cm, I.D. 50 μm) at 25 kV. The optimized background electrolyte was 200 mM borate buffer (pH 9.3, adjusted with triethylamine) containing 10 mM 3-nitrobenzoate as the chromogenic co-ion.	[199]
Simultaneous chromatographic separation of enantiomers, anomers and structural isomers of some biologically relevant monosaccharides.	A one-step chiral high-performance liquid chromatography (HPLC) method was developed to separate anomers and enantiomers of some carbohydrates—glucose, fructose, arabinose, ribose, fucose, mannose, lyxose and xylose, using a Chiralpak AD-H column.	[200]
Fermentation monitoring based on HPTLC–OPLC. The effect of a complex biological matrix on quantitative performance.	An OPLC method has been established for separation and quantification of eight carbohydrates (glucose, fructose, galactose, lactose, sucrose, raffinose, 1-kestose, nystose, and fructosyl-nystose), which might be simultaneously present in a carbon-rich medium to be fermented by a strain of *B. adolescentis*.	[201]
Confirmation and determination of sugars in soft drink products by IEC with ESI-MS.	Ion-exclusion chromatography with electrospray ionization mass spectrometry was investigated as an alternative method to confirm and determine sugar (glucose, fructose and sucrose) using volatile acidic eluent.	[202]
Optimized conditions for 2-aminobenzamide labeling and high-performance liquid chromatography analysis of *N*-acylated monosaccharides.	The separation was achieved on a C_{18} column with a gradient mobile phase composed of water (0.1% formic acid)-methanol (volume varying) in less than 19 min with 12.5 and 18.3 min retention times for GlcNAc and GlcNC16, respectively.	[203]

Table 1.13: Literature appeared during 1995-2009 on chromatography of nucleobases

Title	Remark	Ref.
Nitrogen-specific liquid chromatography detection of nucleotides and nucleosides by HPLC-CLND.	A preliminary study of column, mobile phase, and the HPLC-CLND optimization is presented.	[204]
Automated screening system for purine and pyrimidine metabolism disorders using high-performance liquid chromatography	An automated screening system consisting of a reversed-phase column, a cation-exchange column, a column switch, four sets of ultraviolet absorbance detectors, a microcomputer and other conventional equipment for purine and pyrimidine metabolism disorders is described.	[205]
Improvements in the analytical method for 8-hydroxydeoxyguanosine in nuclear DNA.	Modifications at two points in the sequence of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) analysis have contributed to a more accurate and simplified determination of 8-OH-dG in DNA. The first was an improvement in the detection limit for 8-OH-dG in high-performance liquid chromatography analysis and the second was a pronase digestion and ethanol precipitation method (pronase/ethanol method) for DNA isolation.	[206]
Chromatographic properties of cytosine, cytidine and their synthetic analogues.	A direct RP-HPLC assay was used for the study of the effects of methanol concentration, pH, flow-rate of the mobile phase and column temperature on the retention of the natural nucleic acid components cytosine and cytidine and their synthetic 1-β-D -arabinofuranosyl, 5-aza and 6-aza analogues.	[207]

Title	Remark	Ref.
Separation of twenty isomers of ribonucleotides and deoxyribonucleotides by reversed-phase ion-pairing high-performance liquid chromatography.	A procedure is described for the separation of 20 isomers of ribonucleotides and deoxyribonucleotides using a Waters μBondapak C_{18} 10μm column and a gradient elution system consisting of phosphate buffer (0.05 *M*, pH 5.45) and methanol.	[208]
Chromatographic properties of cytosine, cytidine and their synthetic analogues.	A direct RP-HPLC assay was used for the study of the effects of methanol concentration, pH, flow-rate of the mobile phase and column temperature on the retention of the natural nucleic acid components cytosine and cytidine and their synthetic 1-β-D -arabinofuranosyl, 5-aza and 6-aza analogues.	[209]
Separation of 7-methyl- and 7-(2-hydroxyethyl)-guanine adducts in human DNA samples using a combination of TLC and HPLC.	A combination of thin-layer chromatography (TLC) and high pressure liquid chromatography (HPLC) to achieve separation of ^{32}P-postlabelled 7-methylguanine and 7-(2-hydroxyethyl)-guanine adducts. The chromatographic behaviour and depurination at neutral pH indicated the probable 7-alkylguanine or 3-alkyladenine nature of these unidentified adducts.	[210]
Purification of thymine dimer from monomer solution in preparative liquid chromatography.	Cyclobutane pyrimidine dimers and monomers of thymine were separated on a C_{18} RP-HPLC column using two mathematical models. The effect of the sample sizes on peak shapes in preparative liquid chromatography was investigated.	[211]

Title	Remark	Ref.
Separation of nucleosides and their bases by reversed-phase liquid chromatography using pure water as the mobile phase.	A new RP-LC method for the separation of nucleosides and their bases using pure water as the mobile phase is described.	[212]
Rapid and highly automated determination of adenine and pyridine nucleotides in extracts of *Saccharomyces cerevisiae* using a micro robotic sample preparation-HPLC system.	Microbial extracts including metabolites, macromolecular constituents and inorganic compounds were loaded onto a ODS pre-column in the presence of triethylamine phosphate (TEA-P_i) resulting in a selective binding of the nucleotides and removing of interfering compounds.	[213]
Simultaneous measurement of adenosine and hypoxanthine in human umbilical cord plasma using reversed-phase high-performance liquid chromatography with photodiode-array detection and on-line validation of peak purity.	A new, robust and sensitive RP-HPLC method was developed for concomitant measurement of plasma concentrations of the ATP catabolites adenosine and hypoxanthine in human umbilical cord blood.	[214]
Ion-exchange column chromatographic method for assaying purine metabolic pathway enzymes.	A modified single ion-exchange column chromatographic method using DEAE-Sephadex was used to determine the products of incubation of 5'-NT, AK, APRT and HPRT with their respective substrates.	[215]
Analysis of purine and pyrimidine bases, nucleosides and deoxynucleosides in brain microsamples (microdialysates and micropunches) and cerebrospinal fluid.	Chromatographic peaks were identified via the retention times of known standards, with detection at two wavelengths, and also by electrospray tandem mass spectrometry, which permits the identification of certain compounds at extremely low concentrations.	[216]

Title	Remark	Ref.
High-performance liquid chromatography of some purine and pyrimidine derivatives on silica in hexane–isopropanol–ethylene glycol mobile phases.	An alternative approach for the separation of polar solutes in chromatographic systems consisting of unmodified silica and mobile phases containing solvents with limited mutual solubility has been developed.	[217]
Separation of Pyrimidine Bases on a HPLC Stationary RP-18 Phase Coated with Calix[4]resorcinarene	Lipophilic calix[4]resorcinarenes, derived from lauryl aldehyde and resorcinol, are strongly adsorbed on the modified silica gel RP-18 for HPLC chromatography, while their solutions are passed through the column. The calix[4]resorcinarene-coated RP-18 phases were found to be useful beds for HPLC separation of uracil, thymine and cytosine.	[218]
Simultaneous determination of purine metabolites, creatinine and pseudouridine in ruminant urine by reversed-phase high-performance liquid chromatography.	Separation and quantification was achieved using a Spherisorb ODS II C_{18} column (250×4.6 mm I.D.) under isocratic conditions. The mobile phase contained 7.5 m*M* ammonium dihydrogen phosphate, 10 m*M* sodium 1-heptane sulphonic acid and 1.0 m*M* triethylamine at pH 3.0 at a flow-rate of 1.0 ml/min and monitoring column effluent at 218 nm.	[219]
Separation of purines and pyrimidines by normal-phase high-performance liquid chromatography using dimethyl sulfoxide in binary and ternary eluents.	The substitution of isopropanol by dimethyl sulfoxide in binary eluents results in a specific selectivity of the chromatographic system and shows an improvement of the peak shape for the solutes under study.	[220]
Discontinuous electrophoretic stacking system for cholate-based electrokinetic chromatographic separation of 8-hydroxy-2′-deoxyguanosine from unmodified deoxynucleosides.	Separations of 8-OHdG from 2′-deoxyadenosine, 2′-deoxycytosine, 2′-deoxyguanosine, and thymidine are accomplished using micellar electrokinetic capillary chromatography with sodium cholate.	[221]

Title	Remark	Ref.
Application of tetraphenylporphyrin stationary phases in HPLC of nucleotides and nucleosides.	Interactions of nucleosides and nucleotides with metalloporphyrins have been utilised for HPLC separation of these species with silica-based stationary phases with immobilized tetraphenylporphyrins metallated with Zn (II) and Cu (II).	[222]
Interaction of porphyrin and sapphyrin macrocycles with nucleobases and nucleosides: Spectroscopic, quantum chemical and chromatographic investigation.	Retention behavior of nucleobases and nucleosides was investigated on Novel oligopyrrole macrocycle-based sorbents by HPLC. UV–VIS and ^{1}H NMR titrations were used to study the role of oligopyrrol macrocyclic receptors for selective recognition of adenine, cytosine, thymine and uracil and their nucleoside forms.	[223]
Application of mixed partition–adsorption systems in high-performance liquid chromatography of purines and pyrimidines.	Separation of the test mixtures of some purine and pyrimidine derivatives on silicas (types A and B) in adsorption normal-phase (A-NP) and mixed partition–adsorption normal-phase (MPA-NP) mode has been studied.	[224]
Determination of purine bases in sea urchin (*Paracentortus lividus*) gonads by high-performance liquid chromatography.	Purine bases were separated using a C-18 column as the stationary phase, 0.3 M KH_2PO_4 buffer solution as the mobile phase, and ultraviolet detection at 255 nm.	[225]
Chiral separation of nucleoside analogues of d4T and acyclovir, by liquid chromatography, of amylose stationary phases and determination of enantiomeric purity.	The resolution was accomplished using normal phase methodology with a mobile phase consisting of *n*-hexane-alcohol (ethanol or 2-propanol) in various percentages.	[226]
Purification of 37–38 kDa proteins of barley chloroplast by ammonium sulfate precipitation and heparin-agarose chromatography.	The binding of these proteins to the *in vitro* transcribed chloroplast *psb*A mRNA was established by UV cross-linking assay and SDS-PAGE electrophoresis.	[227]

Title	Remark	Ref.
Determination of purines and pyrimidines in beer samples by capillary zone electrophoresis.	Ten purine and pyrimidine bases were separated using capillary zone electrophoresis (CZE) with direct UV detection at 254 nm as well as mass spectrometric (MS) detection using an electrospray ionization (ESI) interface.	[228]
Separation of purine and its derivatives by capillary zone electrophoresis.	A systematic approach was used to study the effect of pH, buffer type, organic modifiers, applied potential, sodium dodecyl sulfate (SDS) and cyclodextrins on the separation of these purine derivatives.	[229]
Simultaneous determination of purine nucleotides, their metabolites and β-nicotinamide adenine dinucleotide in cerebellar granule cells by ion-pair high performance liquid chromatography.	The chromatographic analysis requires 26 min per sample and allows the separation of the mentioned metabolites in a time as short as 16 min.	[230]
Simultaneous determination of multiple constituents in real beer samples of different origins by capillary zone electrophoresis.	Simultaneous determination of alcohols, amines, amino acids, flavonoids, and purine and pyrimidine bases in bottled beer samples directly without any pre-treatment was carried out by capillary zone electrophoresis with diode-array detection.	[231]
Studies on the chromatographic behavior of nucleosides and bases on *p-tert*-butyl-calix[8]arene-bonded silica gel stationary phase by HPLC.	The chromatographic behavior of some nucleosides, pyrimidines and purines on a new *p-tert*-butyl-calix[8]arene-bonded silica gel stationary phase (CABS) were studied by high performance liquid chromatography.	[232]
Analysis of the Releasable Nucleotides of Platelets	In platelets, two pools of nucleotides have been demonstrated: one is utilized for the metabolic needs of the platelets (*1*) and the second stores nucleotides in a metabolically inert form in dense granules.	[233]

Title	Remark	Ref.
Simultaneous high performance liquid chromatographic separation of purines, pyrimidines, *N*-acetylated amino acids, and dicarboxylic acids for the chemical diagnosis of inborn errors of metabolism.	A novel simple, sensitive, and reliable ion-pairing HPLC method for the synchronous separation of several purines, pyrimidines, *N*-acetylated amino acids, and dicarboxylic acids for the chemical diagnosis and screening of inborn errors of metabolism (IEM).	[234]
Comprehensive analysis of pyrimidine metabolism in 450 children with unspecific neurological symptoms using high–pressure liquid chromatography–electrospray ionization tandem mass spectrometry.	Uracil and thymine as well as their degradation products in urine were determined with an improved method based on reversed-phase HPLC coupled with electrospray ionization-tandem mass spectrometry and detection by multiple-reaction monitoring using stableisotope-labelled reference compounds as internal standards.	[235]
The retention behavior of some uracil derivatives in normal and reversed-phase chromatography. Lipophilicity of the compounds.	Benzene - methanol, benzene - acetonitrile, and benzene-isopropanol were used as mobile phase in normal- phase chromatography and water- methanol and water- acetontrile in reverse phase chromatograhy.	[236]
Determination of total ribonucleotide pool in plant materials by high-pH anion-exchange high-performance liquid chromatography following extraction with potassium hydroxide.	A new, improved method that only requires a potassium hydroxide extraction procedure is presented for the analysis of a full nucleotide pool in plant materials.	[237]
Intracellular nucleotide and nucleotide sugar contents of cultured CHO cells determined by a fast, sensitive, and high-resolution ion-pair RP-HPLC.	A sensitive and reproducible ion-pair RP-HPLC method has been developed, allowing the direct and simultaneous detection and quantification of some essential nucleotides and nucleotide sugars. After a perchloric acid extraction, 13 molecules (8 nucleotides and 5 nucleotide sugars) were separated, including activated sugars such as UDP-glucose, UDP-galactose, GDP-mannose, UDP-*N*-acetylglucosamine, and UDP-*N*-acetylgalactosamine.	[238]

Title	Remark	Ref.
A simple and cost effective method for the quantification of 8-hydroxy-2′-deoxyguanosine from urine using liquid chromatography tandem mass spectrometry.	A rapid and high-throughput method for the determination of urinary levels of the oxidative stress biomarker, 8-hydroxy-2′-deoxyguanosine (8-OH-dG), has been developed and validated using liquid chromatography combined with electrospray ionization tandem mass spectrometry (LC-MS/MS).	[239]
Development and application of a liquid chromatographic method for analysis of nucleotides and nucleosides in milk and infant formulas	A method for the simultaneous determination in milk of the 5′-mononucleotides adenosine 5′-monophosphate, cytidine 5′-monophosphate, guanosine 5′-monophosphate, inosine 5′-monophosphate and uridine 5′-monophosphate and their corresponding nucleosides is described.	[240]
Liquid chromatography-tandem mass spectrometric assay for the nucleoside reverse transcriptase inhibitor emtricitabine in human plasma.	Determination of the antiretroviral nucleoside emtricitabine in human plasma was developed and validated using a simple sample pre-treatment procedure. After addition of 5′-deoxy-5-fluorocytidine as the internal standard and protein precipitation with acetonitrile, the supernatant was directly injected in the isocratic chromatographic system using a polar embedded reversed-phase column and formic acid in water-methanol as the eluent.	[241]
HPLC in biopharmaceutical investigations of drugs representing pyrimidine derivatives (A review)	The analysis of pyrimidine derivatives is typically performed using various chromatographic techniques, in particular, RP-HPLC). The separation is typically carried out with (7–30)-cm-long C_8 and C_{18} silica gel columns, mainly at room temperature, and a 1–1.5 ml-min eluent flow rate.	[242]

Title	Remark	Ref.
Effect of mobile phase additives on resolution of some nucleic compounds in high performance liquid chromatography	RP-HPLC of nucleic compounds (nucleobases, nucleosides, and nucleotides) on a C_{18} column in several different mobile phase additives, including1-butyl-3-methylimidazolium tetrafuloroborate ([BMIm][BF_4]), 1-ethyl-3-methylimidazolium methylsulfate ([EMIm][MS]) ionic liquids, ammonium formate, and potassium phosphate.	[243]
Simple HPLC-UV determination of nucleosides and its application to the authentication of *Cordyceps* and its allies	HPLC-UV method combined with a simple extraction procedure of nucleosides (adenosine, cordycepin, 2′-deoxyadenosine, guanosine and uridine) was developed and applied to the authentication of *Cordyceps* and its allies. The separation was performed on a C_{18} column by isocratic elution with acetonitrile-water, and UV detection at 260 nm.	[244]
Application of Diol column under adsorption and mixed partition–adsorption normal-phase liquid chromatography mode for the separation of purines and pyrimidines.	The diol-bonded phase (column LiChrospher 100 Diol) has been studied for the separation of some purines and pyrimidines under normal-phase liquid chromatography (NPLC) conditions. Retention time, column efficiency, and selectivity of column with diol-phase were compared to those of unmodified silica (column LiChrospher SI-60).	[245]
Quantitative profiling of nucleotides and related phosphate-containing metabolites in cultured mammalian cells by liquid chromatography tandem electrospray mass spectrometry	A method has been developed for the quantitative profiling of over twenty nucleotides and related phosphorylated species using ion-pair reversed-phase liquid chromatography hyphenated to negative ion tandem electrospray mass spectrometry.	[246]

Title	Remark	Ref.
Quantification of Benzo[a]pyrene-Guanine Adducts in *in vitro* Samples by LC Tandem Mass Spectrometry with Stable Isotope Internal Standardization.	A sensitive liquid chromatography/tandem mass spectrometry method coupled with a stable isotope internal standard was developed for detection and quantitative analysis of benzo[a]pyrene with DNA adducts. *In vitro* samples were analyzed by our method. The method provides structural confirmation of the adduct, as well as quantitative analysis with accuracy, confidence, isomeric specificity, and precision to measure biologically relevant levels in small sample sizes.	[247]
Separation and characterization of oxaliplatin dinucleotides from DNA using HPLC-ESI ion trap mass spectrometry.	Using a high-performance liquid chromatography, we carried out the separation of individual platinum-DNA adducts which were concurrently identified using electrospray ionization ion trap mass spectrometry (MS).	[248]
Prediction of the Gradient Retention Times of Purine Compounds in Reversed Phase High Performance Liquid Chromatography.	A numerical method that transforms any gradient condition into discrete step gradient conditions was also proposed to predict the retention times in a gradient elution. The model is suitable to apply to nonlinear and multilinear gradient conditions, including actual obtained gradient profiles. Two kinds of organic modifiers, methanol and acetonitrile, were employed, and four purine compounds were used as solutes.	[249]
Effect of Mobile Phase pH and Organic Content on LC–MS Analysis of Nucleoside and Nucleotide HIV Reverse Transcriptase Inhibitors.	HPLC–MS–MS method for sensitive measurement of FTC, 3TC, and TFV in plasma from macaques. To achieve detection limits of 10 pg on column, the plasma analytes were measured using acidic mobile phase and positive electrospray ionization MS–MS detection.	[250]

Title	Remark	Ref.
An ion-pair reversed-phase HPLC method for determination of fresh tissue adenine nucleotides avoiding freeze–thaw degradation of ATP	An ion-pair reversed-phase HPLC method by which separation of adenine nucleotides can be performed rapidly, allowing multiple analyses in 1 day, with both high sensitivity and extraction efficiency and using fresh samples, thereby avoiding freeze–thaw degradation of nucleotides.	[251]
Simultaneous quantification of free nucleotides in complex biological samples using ion pair reversed phase liquid chromatography isotope dilution tandem mass spectrometry.	A new sensitive and accurate analytical method has been developed for quantification of intracellular nucleotides in complex biological samples from cultured cells of different microorganisms such as *Saccharomyces cerevisiae*, *Escherichia coli*, and *Penicillium chrysogenum*.	[252]
A new, validated HPLC-MS/MS method for the simultaneous determination of the anti-cancer agent capecitabine and its metabolites: 5′-deoxy-5-fluorocytidine, 5′-deoxy-5-fluorouridine, 5-fluorouracil and 5-fluorodihydrouracil, in human plasma.	A rapid and selective liquid chromatography/tandem mass spectrometric method was developed for the simultaneous determination of capecitabine and its metabolites 5′-deoxy-5-fluorocytidine (5′-DFCR), 5′-deoxy-5-fluorouracil (5′-DFUR), 5-fluorouracil (5-FU) and dihydro-5-fluorouracil (FUH_2) in human plasma.	[253]
Determination of purine contents of alcoholic beverages using high performance liquid chromatography.	The method employed in this study is a quantitative determination of purine contents by HPLC. Alcoholic beverages were hydrolyzed to corresponding purine bases, which were then separated by HPLC, and base peaks were identified using an enzymatic peak-shift technique.	[254]

REFERENCES

[1] K. Danzer, Fresenuis, *J. Ana.l Chem.* **343** (1992) 827.

[2] F. Runge, *"Der Biddhungstriebder Stoffe"* Verenschlaulicht in Selbstaqndiggewachsenen Bedern. Sebsterlag Oranenberg, Germany (1855).

[3] F. Goppelsroeder, *Verk Naturforsch, Ces. Basel* **3** (1861) 268.

[4] C. F. Schonbein, *Verk Naturforsch, Ces. Basel* **3** (1861) 249.

[5] E. Fischer, E. Schmidner, *Annalen* **272** (1892) 156.

[6] L. Reed, *Proc. Chem. Soc.* **9** (1893) 123.

[7] D. T. Day, *Amer. Phil. Soc.* **36** (1897) 112.

[8] M. S. Tswett, *Ber. Deut. Boatan. Ges.* **24** (1906) 235.

[9] R. Kuhn, E. Lederer, *Ber.* **64** (1931) 1349.

[10] R. Kuhn, A. Winterstein, E. Lederer, *Hoppe – Seyler'sz. Physiol. Chem.* **197** (1931) 141.

[11] A. J. P. Martin, R. L. M. Synge, *J. Biochem.* **35** (1941) 91.

[12] A. J. P. Martin, R. L. M. Synge, *J. Biochem.* **35** (1941) 1358.

[13] E. Stahl, in *"Thin Layer Chromatography"*, 2nd edn. E. Stahl (ed.) Springer-Verlag, Berlin **1** (1961).

[14] J. G. Kirchner, *J. Chromatogr. Sci.* **13** (1975) 558.

[15] J. G. Kirchner, in *"Thin Layer Chromatography"*, 2nd edn. Wiley-Interscience, New York (1978).

[16] N. Pelick, H. R. Bolliger, H. K. Mangold, in *"Advances in Chromatography"* Vol. 3, J. C. Giddings and R. A. Keller (eds.), Marcel Dekker, New York **85** (1996).

[17] M. W. Beyerink, R. L. M. Synge, *J. Biochem.* **35** (1941) 1358.

[18] H. P. Wijsman, D. De Beshouwdals, *Mengsel Van. Mattase en Dentrinase, Amsterdam* (1898).

[19] N. A. Izmailov, M. S. Schraiber, *Farmatsiya (Sofia)* **3** (1938) 1.

[20] C. Lapp, Erali, *Bull. Sci. Pharmacol.* **47** (1940) 49.

[21] J. E. Meinhard, N. F. Hall, *Anal. Chem.* **21** (1949) 185.

[22] E. P. Przybylowicz, W. J. Staudenmayer, E. S. Perry, A. D. Baitsholt, T. N. Tischer, *J. Chromatogr.* **20** (1956) 506.

[23] H. Halpaap, *J. Chromatogr.* **78** (1973) 77.

[24] A. Zlatkis, R. E. Kaiser, in HPTLC high performance Thin Layer Chromatography, A. Zlatkis, R.E. Kaiser (eds) Elsevier, Amsterdam, Netherlands, **12** (1977).

[25] H. E. Hauck, W. Jost, 'Instrumental High Performance, Thin Layer Chromatography', Proceedings of 2nd International Symposium, R.E. Kaiser (ed), Interlaken, Switzerland, (1982) 25.

[26] H. E. Hauck, W. Jost, 'Instrumental High Performance Thin Layer Chromatography', Proceedings of 3rd International Symposium, R.E. Kaiser (ed), Wu¨ rzburg, Germany (1985) 83.

[27] H. E. Hauck, W. Jost, 'Instrumental High Performance Thin Layer Chromatography', Proceedings of 4th International Symposium, R.E. Kaiser, H. Traitler, A. Studer (eds), Selvino/Bergamo, Italy (1987) 241.

[28] K. Burger, Z. *Anal. Chem.* **318** (1984) 228.

[29] S. Nyiredy, C. A. J. Erdelmeier, O. Sticher, Instrumental Preparative Planar Chromatography, in: "Planar Chromatography, Vol. 1." (ed) by R.E. Kaiser, Heidelberg, Hiithig Verlag.

[30] L. J. Cline Love, J. G. Harbarta, J. G. Dorsy, *Anal. Chem.* **56** (1984) 1132.

[31] S. N. Shtykov, *J. Anal. Chem.* **55** (2000) 608.

[32] D. M. Small, In "The Bile Acids" P. P. Nair, D. Kritchevsky, Eds. Plenum Press: New York, (1971) Chapter 8.

[33] D. W. Armstrong, *Am. Laboratory* **13** (1981) 14.

[34] D. Oakenfull, In "Aggregation Processes in Solution"; E. Wyn-Jones, J. Gormally, Eds.; Elsevier: New York, 1983; Chapter 5.

[35] M. Ash, I. Ash, "Encyclopedia of Surfactants"; Chemical Publishing Co., Inc.: New York, (1981) Volumes I-III; 1985.

[36] J. Moilliet, B. Collie, W. Black, Van Nostrand, Princeton, New Jersey, (1961).

[37] D. W. Armstrong, J. H. Fendler, *Biochim. Biophys. Acta.* **478** (1977) 75.

[38] D. W. Armstrong, R. Q. Terrill, *Anal. Chem.* **51** (1979) 2160.

[39] D. W. Armstrong, M. McNeely, *Anal. Lett.* **12** (1979) 1285.

[40] D. W. Armstrong, G. Y. Stine, *J. Amer. Chem. Soc.* **105** (1983) 2962.

[41] D. W. Armstrong, K. H. Bui, *J. Liq. Chromatogr.* **5** (1982) 1043.

[42] D. W. Armstrong, *J. Liq. Chromatogr.* **3** (1980) 895.

[43] D. W. Armstrong, K. H. Bui, R. M. Barry, *J. Chem. Educ.* **61** (1984) 457.

[44] D. W. Armstrong, S. J. Henry, *J. Liq. Chromatogr.* **3** (1980) 657.

[45] D. W. Armstrong, F. Nome, *Anal. Chem.* **53** (1981) 1662.

[46] D. W.Armstrong, W. L. Hinze, K. H. Bui, H. N. Singh, *Anal. Lett.* **14** (1981) 1659.

[47] D. W. Armstrong, G. Y. Stine, *J. Amer. Chem. Soc.* **105** (1983) 6220.

[48] D. W. Armstrong, G. Y. Stine, *Anal. Chem.* **55** (1983) 2317.

[49] D. W. Armstrong, T. J. Ward, A. Berthod, *Anal. Chem.* **58** (1986) 579.

[50] T. P. Hoar, J. H. Schulman, *Nature* **152** (1943) 102.

[51] C. Solans, H. Kunieda, *Industrial Applications of Microemulsions*, Marcel Dekker, New York, (1997).

[52] Kumar, K. L. Mittal, *Handbook of Microemulsions: Science and Technology*, Marcel Dekker, New York, (1999).

[53] S. P. Moulik, K. Mukherjee, *Proc. ndian Natl. Sci. Acad.* A. **62** (1996) 215.

[54] B. K. Paul, S. P. Moulik, *J. Disp. Sci. Technol.* **18** (1997) 301.

[55] C. Saribu, C. Cimpoiu, T. Hodisan, *Rev. Chim.* **46** (1995) 581.

[56] M. Gui, S.C. Rutan, A. Agbodjan, *Anal. Chem.* **67** (1995), 3293.

[57] Y. Wu, B. Kuang, F. Long, *Sepu.* **14** (1996) 259.

[58] R. Bhushan, I. Ali, S. Sharma, *Biomed. Chromatogr.* **10** (1996) 37.

[59] M. B. Huang, H. K. Li, G. L. Li, C.T. Yan, L. P. Wang, *J. Chromatogr. A.* **742** (1996) 289.

[60] I. I Malakhova, B.V. Tyaglov, E. V. Degterev, V. D. Krasikov, W. G. Degtiar, *J. Planar Chromatogr.- Mod. TLC* **9** (1996) 375.

[61] G. Matsik, *Chromatographia,* **43** (1996) 39.

[62] D. Guo, X. Xeng, J. Yang, *Zhongguo Shenghua Yaowu Zazhi* **17** (1996) 119.

[63] J. Weng, J. Qiu, Y. Zheng, S. Fujian, *Daxue Xuebao Ziran Kaxue Ban* **12** (1996) 31.

[64] A. Makriyannis, A. A. El Khair, M. M. Ayad, W. S. Hassan, M. M. F. Metwally, *Spectrosc. Lett.* **11** (1997) 286.

[65] I. Malinowska, J. K. Rozylo, *Biomed. Chromatogr.* **11** (1997) 272.

[66] R. Bhushan, J. Martens, S. Wallbaum, S. Joshi, V. Prasad. *Biomed. Chromatogr.* **11** (1997) 286.

[67] Q. Y. Deng, Z. Zhang, J. Y. Su, *Chin. Chem. Lett.* **8** (1997) 161.

[68] A. Pyka, *J. Liq. Chromatogr. Related Technol.* **22** (1999) 41.

[69] W. G. Degtiar, B. V. Tyaglov, E. V. Degterev, V. M. Krylov, I. I. Malakhova, V. D. Krasikov, *J.Planar Chromatogr.-Mod.TLC* **13** (2000) 217.

[70] E. V. Degterev, W. G. Degtiar, B. V. Tyaglov, A. P. Tarasov, V. M. Krylov, I. I. Malakhova, V. D. Krasikov, *J.Planar Chromatogr.-Mod TLC* **13** (2000) 191.

[71] A. Mohammad, V. Agrawal, *J.Planar Chromatogr.-Mod. TLC* **11** (2000) 171.

[72] S. Wawrzycki, E. Pyra, B. Wawrzycki, *Herba Polonica* **18** (2000) 329.

[73] D. T. Tian, Y. M. Dan, H. Wang, L. Z. Wang, H. Q. Zie, *Yingyong Huaxue*, **18** (2001) 329.

[74] R. Bhushan, M. Arora, *Biomed.Chromatogr.* **15** (2000) 433.

[75] A. Mohammad, V. Agrawal, *Indian J. Chemistry* **40** (2001) 1130.

[76] S. D. Sharma, H. Sharma, S. C. Sharma, *J. Indian Chem. Soc.* **78** (2001) 419.

[77] S. D. Sharma, H. Sharma, S. C. Sharma, *Chem.Environ. Res.* **11** (2002) 179.

[78] A. Mohammad, Y. H. Sirwal, V. Agrawal, *Acta Universitatis Cibiniensis, Seria F: Chemia* **5** (2002) 37.

[79] D. Tian, B. Shi, Y. Xie, *Sepu.* **21** (2003) 91.

[80] A. J. Mota, E. Castellanos, E. Juaristi, *Org. Prep. Proc. Int.* **35** (2003) 414.

[81] S. Khawas, S. Laskar, *J. Planar Chromatogr.-Mod. TLC* **16** (2003) 165.

[82] R. Zakrzewski, W. Ciesielski, D. Kazmierezak, *J. Sepn. Sci.* **26** (2003) 1063.

[83] S. A. Nabi, M. A. Khan, *Acta Chromatogr.* **13** (2003) 161.

[84] G. Cao, Y. Zheng, C. Xu, L. Wang, *Zhongguo Yaoxue Zazhi* **38** (2003) 52.

[85] S. D. Sharma, H. Sharma, C. Sharma, *J. Indian Chem. Soc.* **80** (2003) 926.

[86] I. L. Drozdova, R.A. Bubenchikov, *Farmatsiya* **5** (2003) 14.

[87] A. I. Oliva, L. Simon, F. M. Muniz, F. Sanz, C. Ruiz-Valero, J. R. Morgan, *J. Org. Chem.* **69** (2004) 6883.

[88] T. D. Samanta, M. Nag, S. Laskar, *Asian J. Chem.* **16** (2004) 1947.

[89] Z. G. Randic, *Farmaceutski Glasnik* **60** (2004) 381.

[90] S. Khawas, D. Panja, S. Laskar. *J. Planar Chromatogr.-Mod. TLC* **17** (2004) 314.

[91] T. Cserhati, E. Forgacs, *Amino Acids*, **28** (2005) 99.

[92] D. Kazmirczak, W. Ciesielski, R. Zakrzewski, *J. Planar Chromatogr.-Mod. TLC*, **18** (2005) 427.

[93] J. Sherma, B. J. Hess, *International Scientific Communication, Inc.* **37** (2005) 28.

[94] A. Mohammad, H. Shahab, S. Hena, *Anal. Agr. Sci.* **3** (2005) 7.

[95] B. Basak, D. Bandyopadhyay, M. Patra, A. Banerji, A. Chatterjee, J. Banerji, *J. Chromatogr. Sci.* **43** (2005)104.

[96] A. Mohammad, S. Hena, S. A. Bhawani, *Ind. J. Chemistry* A **45** (2006) 1663.

[97] A. Mohammad, A. Zehra, *Acta Universitatis Cibiniensis Seria F: Chemia*, **9** (2006) 17.

[98] T. D. Samanta, S. Laskar, *J. Planar Chromatogr. -Mod. TLC* **19** (2006)252.

[99] J. Flieger, M. Tatarczak, H. Szumito, *J. Planar Chromatogr. -Mod. TLC* **19** (2006) 161.

[100] B. Polak, W. Golkiewicz, T. Tuzimski, *Chromaographia* **63** (2006)197.

[101] A. Mohammad, A. Zehra, *Colloids and Surfaces A* **301** (2007) 404.

[102] A. Mohammad, S. Laeeq. *J. Planar Chromatogr. -Mod. TLC* **20** (2007) 423.

[103] A. Mohammad, A. Zehra, *Proc. Nat. Acad. Sci.* **78** (2008)11.

[104] A. Mohammad, A. Zehra, *J. Planar Chromatogr.* **21** (2008) 299.

[105] A. Mohammad, R. Gupta, *Colloids and Surfaces B* **65** (2008)166.

[106] M. Zaher, C. Ravelet, I. Baussanne, A. Ravel, C. Grosset, J-l Decout, E.Peyrin, *Anal. Bioanal. Chem.* **393** (2009) 655.

[107] R. Bhushan. V. Kumar, S. Tanvar, *Amino acids* **36** (2009) 571.

[108] S.Preet, S.Gupta, *Res. J. Parasitol.* **4** (2009) 87.

[109] A. Mohammad, N. Haq, *Der Pharma Chemica* **1** (2009) 113.

[110] P. Janjic, N. V. Poponic, M. R. Diakovic, T. Li, *Acta Chromatographica* **5** (1995) 144.

[111] I. Baranowska, A. Kadziolka, *Acta Chromatographica* **6** (1996) 61.

[112] M. W. Roomi, C. S. Tsavo.,*J. Agric Food Chem.* **46** (1998) 1406.

[113] B. B. Petrovska, L. P. Tozi, *Int. J. Food. Sci. Technol.* **35** (2000) 201.

[114] T. Aburjai, B. I. Amro, K. Aiedeh, M. Abuirjeie, S. al-khalil, *Pharmazie* **55** (2000) 751.

[115] R. Bhushan, M. Arora, *Nat. Acad. Sci. Let.* **25** (2002) 159.

[116] K. Tyrpien, R. R. Schefer, S. Bachmann, K. Albert, *J. Planar Chromatogr. -Mod. TLC* **16** (2003) 256.

[117] V. M. Scussel, *Ciencia e Technologia de Alimentos* **23** (2003) 46.

[118] E. A. Illarionova, I. P. Syrovatskii, *Zavodskaya Laboratoriya, Diagnostika Materialov* **69** (2003) 16.

[119] A. Pyka, J. Sliwiok, A. Niestroj, *Acta Poloniae Pharmaceutica* **60** (2003) 327.

[120] E. L. Ponder, J. Sherma, *Acta Universitatis Cibiniesis, Seria F: Chemia*, **7** (2004) 3.

[121] I. A. Ansari, F. H. M. Vaid, I. Ahmad. *Pakistan Journal of Pharmaceutical Sciences* **17** (2004) 19.

[122] M. Georgescu, R. Dinica, B. Furdui, D. Koprivec, *Sepu.* **22** (2004) 256.

[123] E. L. Ponder, B. Fried, J. Sherma, *Acta Chromatographica* **14** (2004) 70.

[124] A. Pyka, W. Klimczok, *J. Planar Chromatography- Mod. TLC* **18** (2005) 300.

[125] F. Buhl, B. Szpikowska- Sroka, M. Galkowska, *J. Planar Chromatogr. - Mod. TLC* **18** (2005) 368.

[126] B. Kivcak, S. Akay, *Fitoterapia* **76** (2005) 62.

[127] C. Cimpoiu, S. Hodisan, R. Bricue, *Acta Universitatis Cibiniensis, Seria F. Chemia*, **8** (2005) 13.

[128] V. M. Vostokov, V. R. Kartashov. I. Khimiya, *Khimicheskaya Tekhnologiya* **49** (2006) 115.

[129] A. M. Hossu, C. Radulescu, M. I. D. Balalau, V. Magearu, *Revista de chimie*, **57** (2006) 1188.

[130] H. Yang, Y. Zhao, Y. Zhang, *Shuli Yiyaoxue Zazhi*, **19** (2006) 536.

[131] L. A. Kartsova, O. A. Koroleva, *J. Anal. Chem.* **62** (2007) 255.

[132] A. Pyka, W. Klimczok, *J. Liq. Chromatogr. Related Technol.* **30** (2007) 3107.

[133] A. Mohammad, S. Laeeq, *The Icfai J. Chem.* **1** (2008) 19.

[134] A. Pyka, W. Klimczok, *J. Liq. Chromatogr. Related Technol.* **31** (2008) 526.

[135] A. Mohammad, R. Gupta, S.A. Bhawani, *Tenside Surfact. Deterg.* **46** (2009) 267.

[136] A. Pyka. *J. Planar Chromatogr. –Mod. TLC* **22** (2009) 211.

[137] A. Mohammad, A. Zehra, *J. Planar Chromatogr. –Mod. TLC* **22** (2009) 1.

[138] B. V. Kharbade, G. P. Joshi, *Stud. Conserv.* **40** (1995) 93.

[139] J. Prodolliet, E. Bugner, M. Feinberg, *J. AOAC Int.* **78** (1995) 768.

[140] C. Corradini, G. Huber, G. K. Bonn, *Chromatographia* **41** (1995) 511.

[141] K. Kano, K. Takagi, K. Inoue, T. Ikeda, T. Ueda, *J. Chromatogr.* A **721** (1996) 53.

[142] S. Yamauchi, T. Hanai, J. Suzuki, M. Ito, Y. Sano, R. Shibata, T. Kinoshita, M. Yaginuma, K. Kadowaki, Y. Takahashi, *J. Chromatogr. A* **737** (1996) 149.

[143] S. C. Churms, *J. Chromatogr. A* **720** (1996) 151.

[144] J. Sherma, D. L. Zulick, *Acta Chromatographica* **6** (1996) 7.

[145] G. Lodi, C. Bighit, V. Brandolini, E. Menziani, B. Tosi, *J. Planar Chromatogr. Mod. - TLC* **10** (1997) 31.

[146] D. S. Lee, C. Wu, H. H. Hill, *J. Chromatogr. A* **822** (1998) 1.

[147] T. L. Niederhauser, J. Halling, N. A. Polson, J. D. Lamb, *J. Chromatogr. A* **804** (1998) 69.

[148] S. M. Anderton, L. R. Layman, J. Sherma, *J. Liq. Chromatogr. Related Technol.* **21** (1998) 1045.

[149] H. Lidén, T. Buttler, H. Jeppsson, G. Marko-Varga, J. Volc, L. Gorton, *Chromatographia* **47** (1998) 501.

[150] D. J. Cline, B. Fried, J. Sherma, *Acta Chromatographica* **9** (1999) 79.

[151] E. E. Mulle, B. Fried, J. Sherma, *J. Chem. Ecol.* **25** (1999) 727.

[152] F. J. Moreno, A. Olano, G. Santa-María, N. Corzo, *Chromatographia* **50** (1999) 705.

[153] J. A. Gómez Bárez, R. J. Garcia-Villanova, S. Elvira Garcia, A. M. González Paramás, *Chromatographia* **50** (1999) 461.

[154] M. E. Quigley, G. J. Hudson, H. N. Englyst, *Food Chem.* **65** (1999) 381.

[155] K. B. Kalinina, L. S. Litvinova, J. Planar *Chromatogr. Mod TLC* **12** (1999) 190

[156] S. Nojiri, N. Taguchi, M. Oishi, S. Suzuki *J. Chromatogr. A* **893** (2000) 195.

[157] T. Soga, M. Serwe, *Food Chem.* **69** (2000) 339.

[158] R. Bhushan, S. Kaur, *Biomed. Chromatogr.* **11** (1998) 59.

[159] R. Andersen, A. Sørensen, *J. Chromatogr. A* **897** (2000)195.

[160] A. Cáceres, S. Cárdenas, M. Gallego, A. Rodríguez, M. Valcárcel, *Chromatographia* **52** (2000) 314.

[161] L. W. Doner, *Chromatographia* **53** (2001) 579.

[162] F. J. Moreno, M. Villamiel, R. López-Fandiño, A. Olano, *Chromatographia* **53** (2001) 525.

[163] J. A. Wasylnka, M. I. Simmer, M. M. Moore, *Microbiol.* **147** (2001) 869.

[164] G. Vaccari, G. Lodi, E. Tamburini, T. Bernardi, S. Tosi, *Food Chem.* **74** (2001) 99.

[165] V. Pitthard, P. Finch, *Chromatographia* **53** (2001) 317.

[166] H. Szumiło, J. Flieger, *Acta Po.l Pharm.* **58** (2001) 3.

[167] J. Flieger, H. Szumilo, *J. Planar Chromatogr.-Mod TLC* **14** (2001) 338.

[168] G. Grygierczyk, W. Fischer, M. Sajewicz, P. Kuś, R. Wrzalik, M. Czaja, M. Dziadek, T. Kowalska, *J. Planar Chromatogr. Mod-TLC* **15** (2002) 449.

[169] T. Masuda, K. Kitahara, Y. Aikawa, S. Arai, *J. Chromatogr.* A **961** (2002) 89.

[170] B. B. Castells, V. C. Arias, R. C. Castells, *Chromatographia* **56** (2002) 153.

[171] L. Zhang, J. Xu, L. Zhang, W. Zhang, Y. Zhang, *J. Chromatogr.* B **793** (2003) 159.

[172] X. Cheng, L. A. Kaplan, *J. Chromatogr. Sci.* **41** (2003) 434.

[173] I. Vovk, B. Simonovska, L. Kompan , M. Prošek, J. Planar Chromatogr. Mod-TLC **16** (2003) 374.

[174] X. Di, K. K. C. Chan, H. W. Leung, C. W. Huie, *J. Chromatogr. A* **1018** (2003) 85.

[175] K. Reiffová, A. Oriňák, R. Nemcová, F. Vreven, J. Planar Chromatogr. –Mod. TLC **16** (2003) 192.

[176] K. Reiffová, J. Podolonovičová, A. Oriňák, K. Flórián, T. Gondová, J. Planar Chromatogr. – Mod TLC 16 (2003) 52.

[177] J. Yang, Y. Sun, J. Yanhua, G. Yang, M. Ting, *Sepu.* **21** (2003) 427.

[178] B.Martínez Montero, M.C. Rodríguez Dodero, D.A. Guillén Sánchez, C.G. Barroso, *Chromatographia* **59** (2004) 15.

[179] M. Mecozzi, Z. Pápai, *J. Chromatogr. Sci.* **42** (2004) 268.

[180] S. Cortacero-Ramírez, A. Segura-Carretero, C. Cruces-Blanco, M. Hernáinz-Bermúdez de Castro, A. Fernández-Gutiérrez, *Food Chem.* **87** (2004) 471.

[181] Mario B. Aranda, Mario H. Vega, Ricardo F. Villegas, *J. Planar Chromatogr. – Mod. TLC* **18** (2005) 285.

[182] C. Guignard, L. Jouve, M. B. Bogéat-Triboulot, E. Dreyer, J. F. Hausman, L. Hoffmann, *J. Chromatogr. A* **1085** (2005) 137.

[183] J. Tiihonen, E. L. Peuha, M. Latva-kokko, S. Silander, E. Paatero, *Sep. Purif. Technol.* **44** (2005) 166.

[184] T. Bernardi, E. Tamburini, G. Vaccari, *J. Planar Chromatogr. – Mod. TLC* **18** (2005) 23.

[185] L. Liang, P. Zhang, Y. Cai, S. Mou, *Chin. J. Anal. Chem.* **34** (2006) 1371.

[186] M. Murkovic, K. Derler, *J. Biochem. Bioph. Methods* **69** (2006) 25.

[187] M. Rossi, C. Campa, A. Gamini, A. Coslovi, I. Donati, A. Vetere, S. Paoletti, *J. Chromatogr. A* **1110** (2006) 125.

[188] T. Barboni, N. Chiaramonti, *Chromatographia* **63** (2006) 445.

[189] A. Montilla, J. Van De Lagemaat, A. Olano, M. D. Del Castillo,

Chromatographia, **63** (2006) 453.

[190] V. Morales, M. L. Sanz, A. Olano, N. Corzo, *Chromatographia* **64** (2006) 233.

[191] F. Momenbeik, J. H. Khorasani, *Acta Chromatographica* **16** (2006) 58.

[192] E. Tamburini, T. Bernardi, M. Granini, G. Vaccari, *J. Planar Chromatogr. – Mod. TLC* **19** (2006) 10.

[193] A. Caseiro, I. L. Marr, M. Claeys, A. Kasper-Giebl, H. Puxbaum, C. A. Pio, *J. Chromatogr. A* **1171** (2007) 3.

[194] T. Wang, P. K. Nam, H. Shi, Y. Ma, *J. Chromatogr. Sci.* **45** (2007) 200.

[195] S. Inagaki, J. Z. Min, T. Toyooka, *Biomed. Chromatogr.* **21** (2007) 4338.

[196] M. L. Sanz, I. Martínez-Castro, *J. Chromatogr. A* **1153** (2007) 74.

[197] J. Li, M. Chen, Y. Zhu, *J. Chromatogr. A* **1155** (2007) 50.

[198] J. Z. Min, T. Toyooka, T. Kurihara, M. Kato, T. Fukushima, S. Inagaki, *Biomed. Chromatogr.* **21** (2007) 852.

[199] M. Pospíšilová, M. Polášek, J. Šafra, I. Petriška, *J. Chromatogr. A* **1143** (2007) 258.

[200] J. F. Lopes, E. M. Gaspar, *J. Chromatogr. A* **1188** (2008) 34.

[201] E. Tamburini, T. Bernardi, E. Bianchini, P. Pedrini, *J. Planar Chromatogr.- Mod. TLC* **22** (2009) 9.

[202] Z. Chen, X. Jin, Q. Wang, Y. Lin, L. Gan, *Chromatographia* **69** (2009) 761.

[203] D. Maury, F. Couderc, J. Czaplicki, J. C. Garrigues, V. Poinsot, *Biomed. Chromatogr.* **24** (2009) 343.

[204] E. M. Fujinari, J. D. Manes *Develop. Food Sci.* **37** (1995) 379.

[205] S. Adachi, M. Zeisig, L. Möller, *Carcinogenesis* **16** (1995) 253.

[206] S. Sumi, K. Kidouchi, S. Ohba, Y. Wada, *J. Chromatogr. B* **672** (1995) 233.

[207] D. Romanová, L. Novotný, *J. Chromatogr. B* **675** (1996) 9.

[208] J. Zhao, G. H. Fleet, *J. Chromatogr. A* **732** (1996) 271.

[209] D. Romanová, L. Novotný *J. Chromatogr. B* **675** (1996) 9.

[210] R. Kumar, K. Hemminki, *Carcinogenesis* **17** (1996) 485.

[211] K. H. Row, *Chromatographia* **45** (1997) 296.

[212] W. Hu, K. Hasebe, D. M. Reynolds, H. Haraguchi, *Analytica Chimica Acta* **353** (1997) 143.

[213] W. Mailinger, A. Baumeister, M. Reuss, M. Rizzi, *J. Biotechnology* **63** (1998) 155.

[214] M. H. Maguire, I. Szabó, I. E. Valkó, B. E. Finley, T. L. Bennett, *J. Chromatogr. B* **707** (1998) 33.

[215] H. Ward, D. Baldwin, T. Wang, H. Warner, K. Seymour, C. Marquardt, E. McFalls, J. E. Foker *J. Chromatogr. B* **707** (1998) 295.

[216] Á. Dobolyi, A. Reichart, T. Szikra, N. Szilágyi, A. K. Kékesi, T. Karancsi, P. Slégel, M. Palkovits, G. Juhász *Neurochemistry International* **32** (1998) 247.

[217] H. Kazoka, *J. Chromatogr. A* **836** (1999) 235.

[218] O. Pietraszkiewicz, M. Pietraszkiewicz, *J. Inclusion Phenomena and Macrocyclic Chemistry* **35** (1999) 261.

[219] K. J. Shingfield, N. W. Offer, *J. Chromatography B* **723** (1999) 81.

[220] H. Kazoka *J. Chromatogr. A* **874** (2000) 45.

[221] T. G. Strein, D. Morris, J. Palmer, J. P. Landers, *J. Chromatogr.* B **763** (2001) 71.

[222] M. Biesaga, F. Kilár, N. Hartvig, M. Trojanowicz, *Chromatographia* **54** (2001) 619.

[223] K. Záruba, Z. Tománková, D. Sýkora, J. Charvátová, I. Kavenová, P. Bour, P. Matejka, J. Fähnrich, K. Volka, V. Král, *Anal. Chim. Acta* **437** (2001) 39.

[224] H. Kazoka, *J. Chromatogr. A* **922** (2001) 187.

[225] M. P. Sotelo, A. R. Bernaldo de Quirós, J. L. Hernández, J. S. Lozano, *Food Chem.* **79** (2002) 113.

[226] E. Lipka-Belloli, V. Glaçon, G. Mackenzie, D. Ewing, C. Len, C. Vaccher and J. P. Bonte, *Chromatographia* **56** (2002) 567.

[227] A. R. Memon, D. Aktoprakligil, *Biotech. Lett.* **24** (2002) 325.

[228] C. W. Klampfl, M. Himmelsbach, W. Buchberger, H. Klein, *Anal. Chim. Acta* **454** (2002) 185.

[229] Z. Bai-zhao, Z. Fa-qiong , *Wuhan Univ. J. Nat. Sci.* **8** (2003) 437.

[230] S. Giannattasio, S. Gagliardi, M. Samaja, E. Marra, *Brain Research Protocols* **10** (2003) 168.

[231] S. Cortacero-Ramírez, A. Segura-Carretero, C. Cruces-Blanco, M. Luisa Romero-Romero, A. Fernández-Gutiérrez, *Anal. Bioanal. Chem.* **380** (2004) 831.

[232] L. S. Li, M. Liu. S. L. Da, Y. Q. Feng, *Talanta* **63** (2004) 433.

[233] J. Jankowski, L. Henning, H.Schlüter, *Methods Mol. Bio.* **272** (2004) 97.

[234] B.Tavazzi, G. Lazzarino, P. Leone, A. M.Amorini, F. Bellia, C. G. Janson, V. D. Pietro, L. Ceccarelli, S. Donzelli, J. S. Francis, B. Giardina, *Clin. Biochem.* **38** (2005) 997.

[235] C. Schmidt, U. Hofmann, D. Kohlmüller, T. Mürdter, U. M. Zanger, M. Schwab, G. F. Hoffmann, *J. Inherit. Metabolic Disease* **28** (2005)

1109.

[236] N. U. Perišić-Janjić, G. S. Usčumlić, N. V. Valentić, *J. Planar Chromatogr.- Mod. TLC* **18** (2005) 92

[237] C. Riondet, S. Morel, G. Alcaraz, *J. Chromatogr. A*, **1077** (2005) 120.

[238] N. Kochanowski, F. Blanchard, R. Cacan, F. Chirat, E. Guedon, A. Marc, J. L. Goergen, *Anal. Biochem.* **348** (2006) 243.

[239] B. Crow, M. Bishop, K. Kovalcik, D. Norton, J. George, J. A. Bralley *Biomed. Chromatogr.* **22** (2007) 394.

[240] B. D. Gill, H. E. Indyk, *Intern. Dairy J.* **17** (2007) 596.

[241] R. W. Sparidans, J. M. Prins, J. H. M. Schellens, J. H. Beijnen *Biomed. Chromatogr.* **21** (2007) 621.

[242] D. V. Moiseev, S. I. Marchenko, A. M. Moiseeva, T. V. Trukhacheva, P. T. Petrov, A. I. Zhebentyaev, *Pharm. Chem. J.* **41** (2007) 25.

[243] C. H. Jin, Y. M. Koo, D. K. Choi, K. H. Row, *Biotech. Biopro. Engg.* **12** (2007) 525.

[244] R. Ikeda, M. Nishimura, Y. Sun, M. Wada, K. Nakashima, *Biomed. Chromatogr.* **22** (2008) 630.

[245] H. Kazoka, *J. Chromatogr. A* **1189** (2008) 52.

[246] R. L. Cordell, S. J. Hill, C. A. Ortori, D. A. Barrett, *J. Chromatogr. B* **871** (2008) 115.

[247] P. C. Chiang, J. C. Means, *J. Liquid Chromatogr. Related Technol.* **31** (2008) 3169.

[248] S. Mowaka , M. Linscheid, *Anal. Bioanalyt. Chem.* **392** (2008) 819.

[249] J. W. Lee, K. H. Row, *J. Liq. Chromatogr. Related Technol.* **31** (2008) 2401.

[250] Z. Kuklenyik, A. Martin, C. P. Pau, J. G. Garcia-Lerma, W. Heneine, J. L. Pirkle, J. R. Barr, *J. Chromatogr. Sci.* **47** (2009) 365.

[251] S. Nedden, R. Eason, A. S. Doney, B. G. Frenguelli, *Anal. Biochem.* **388** (2009)108.

[252] R. M. Seifar, C. Ras, J. C. van Dam, W. M. van Gulik, J. J. Heijnen, W. A. van Winden, *Anal.l Biochem.* **388** (2009) 213.

[253] L. D. Vainchtein, H. Rosing, J. H. M. Schellens, J. H. Beijnen, *Biomed. Chromatogr.* **24** (2009) 4374.

[254] K. Kaneko, T. Yamanobe, S. Fujimori, *Biomed. Chromatogr.* **23** (2009) 858.

CHAPTER-2

Mixed Surfactants Enable Separation of Lysine from other Essential Amino Acids in TLC on Silica Gel

2.1 INTRODUCTION

The urgent need for rapid and selective methods for separation and identification of amino acids has promoted the development of a variety of chromatographic systems for this purpose. The high efficiency, reasonable resolving power, capability of simultaneous analysis of a large number of samples and simple nature of Thin Layer Chromatography (TLC) has resulted in its widespread use for analysis of closely related amino acids.

Numerous TLC systems effective in separating amino acids from each other have been well documented in literature [1-10]. Most literature reports of TLC methods describe use of traditional aqueous, non-aqueous and mixed - aqueous organic solvents as mobile phases. The use of certain organic solvents such as benzene, pyridine, carbon tetrachloride, chloroform, methanol, etc., has been a shortcoming of TLC. As an alternative to complex organic mobile phase systems, in 1979 Armstrong and Terrill introduced micellar mobile phases containing aqueous solutions of surfactants for the separation of pesticides, decachlorobiphenyl and nucleoside [11].

Since then, the importance of surfactant – containing mobile phases in chromatographic separations [12-22] has gained impetus. Organic modifiers (e.g. short chain alcohols) were subsequently added to micellar mobile phases to improve separation selectivity [23-24].

According to literature survey, much work has been reported on the use of aqueous solutions containing single surfactant, with or without added organic modifiers, as mobile phase in TLC analysis of inorganic and organic substances [25-30]. Recently the usefulness of mixed surfactants systems containing ionic - nonionics or mixed-ionic micelles has been realized by physical chemists [31-36]. Synergism is often observed in the physicochemical properties of mixed surfactant and sometimes the performance of mixtures is superior to that of pure surfactant compared to the pure surfactant components. Taking into account the excellent

performance of aqueous solute ions of mixed surfactants, this study was undertaken to examine the resolving power of mixed aqueous surfactants with added acetone in the analysis of amino acids. As a result, we have identified a new TLC system comprising of silica gel as stationary phase and 1.0 x 10^{-5} M Triton X-100 (t-octylphenoxypolyethoxy ethanol) plus 8.1 x 10^{-4} M SDS (sodium dodecyl sulphate) - acetone (1:1:5 v/v/v) as mobile phase for specific separation of lysine from other essential amino acids.

2.2 EXPERIMENTAL

All the experiments were performed at 30 ± 2°C

2.2.1 *Apparatus*

A TLC applicator (Toshniwal, India) was used for coating silica gel G on 20 cm x 3.5 cm glass plates. The chromatography was performed in 24 cm x 6 cm glass jars. A glass sprayer was used to spray reagent on the plates to locate the position of the spot of analyte.

2.2.2 *Reagents*

Sodium dodecyl sulphate(SDS) was from BDH, India. Triton X-100 (Iso-octylphenoxy polyethoxyethanol), abbreviated TX-100 in this paper, and the essential amino acids L-lysine (L-Lys), L-valine (L-Val), L-isoleucine (L-Ile), DL – threonine (DL-Thr), L-methionine (L-Met), L-leucine (L-Leu), DL-phenylalanine (DL-Phe) and DL-tryptophan (DL-Trp) were from CDH, India. Acetone and butan-2-one (Qualigens, India). Ninhydrin from Merck, India was used. All other reagents were of analytical reagent grade.

2.2.3 *Chemical solutions*

Test solutions of amino acids (1%) were prepared in doubled distilled water (DDW). Solutions (1%) of the cations Hg^{2+}, Pb^{2+}, Cu^{2+}, Th^{4+}, Zn^{2+}, Fe^{3+}, Cd^{2+}, Cr^{6+},

Ni^{2+}, Co^{2+}, Mo^{6+}, Al^{3+} and Tl^{3+} were prepared from the metal chlorides, nitrates, or sulphates and contain a small amount of corresponding acid to prevent hydrolysis. Aqueous solution (1%) of the amines, naphthylamine, methylamine, diphenylamine, tri-n-butylamine, tert.-butylamine, and m-phenylenediamine were prepared in methanol. Drug samples were dissolved in 10ml DDW.

Aqueous solution of TX-100 and SDS were prepared in DDW. The concentrations of the surfactants were kept below their critical micelle concentration (CMC; 1.0 x 10^{-4} M for TX 100 and 8.1 x 10^{-3} M for SDS).

2.2.4 *Detector*

Ninhydrin solution (0.3%) in acetone was used to detect all the amino acids.

2.2.5 *Stationary phase*

Silica gel G (Merck, India Batch No.MK6M562890).

2.2.6 *Mobile phases*

Following solvent systems were used as mobile phase

Symbol	Composition
M_1	Double distilled water (DDW)
M_2	Acetone
M_3	DDW + acetone 1:1 (v/v)
M_4	DDW + acetone 1:5 (v/v)
M_5	1.0 x 10^{-5} M TX – 100
M_6	8.1 x 10^{-4} M SDS)
M_7	1.0 x 10^{-5} M TX – 100 + acetone 1:5 (v/v)
M_8	8.1 x 10^{-4} M SDS + acetone 1:5 (v/v)
M_9	1.0 x 10^{-3} M TX – 100 + 8.1 x 10^{-2} M SDS 1:1(v/v)
M_{10}	1.0 x 10^{-5} M TX – 100 + 8.1 X 10^{-4} M SDS 1:1(v/v)
M_{11}	1.0 x 10^{-5} M TX– 100 + 8.1 x 10^{-4} M SDS + acetone 1:1:1(v/v/v)
M_{12}	1.0 x 10^{-5} M TX– 100 + 8.1 x 10^{-4} M SDS + acetone 1:1:2 (v/v/v)
M_{13}	1.0 x 10^{-5} M TX–100 + 8.1 x 10^{-4} M SDS + acetone 1:1:3 (v/v/v)
M_{14}	1.0 x 10^{-5} M TX– 100 + 8.1 x 10^{-4} M SDS + acetone 1:1:5 (v/v/v)
M_{15}	1.0 x 10^{-5} M TX– 100 + 8.1 x 10^{-4} M SDS + acetone 2:1:1 (v/v/v)
M_{16}	1.0 x 10^{-5} M TX– 100 + 8.1 x 10^{-4} M SDS + acetone 3:1:1 (v/v/v)
M_{17}	1.0 x 10^{-5} M TX –100 + 8.1 x 10^{-4} M SDS + butan – 2 – one 1:1:5 (v/v/v)

2.2.7 *Preparation of TLC plates*

Silica gel G was mixed with DDW in the ratio 1:3 and shaken until a plates homogenous slurry was obtained. This slurry was immediately coated as 0.25mm layers on 20 cm x 3.5 cm glass plates by the use of a Toshniwal (India) TLC applicator. The plates were dried in air at room temperature and then activated by heating at 100 ± 2 °C for 1 hour in an electrically controlled oven. After activation the plates were cooled to room temperature than stored in a close chamber at 30 °C before use.

2.2.8 *Procedure*

Test solutions (0.2 µl) were applied to the plates, approximately 2 cm above the lower edge, by means of micropipette (Tripette 0783178, Germany).The spots were dried at room temperature (30 °C) than chromatography was performed on 24 cm x 6 cm glass jars with lids. The various mobile phases are investigated. Chambers were saturated with mobile phase vapor for 10 min .before introduction of the plates. Plates were developed by the ascending technique; the development distance was always 10 cm. After development the plates were withdrawn from the jars and dried in room temperature. A glass sprayer was then used to apply ninhydrin and the plates were than heated at 70 °C to locate the positions of the analyte spot. R_L (R_F of leading edge) and R_T (R_F of trailing edge) values were determined for detected spots and the RF values for amino acids were calculated by the use of the formula

$$R_F = 0.5\ (R_T + R_L)$$

For selective separation, equal volumes (1 ml) of all the essential amino acids solution were mixed and 0.2 µl of the resulting mixture was applied to on an activated TLC plate. The plate was developed with mobile phase M_{14}, the spots were detected and the R_F values of the separated lysine and the other amino acids were calculated.

The limit of detection of lysine was determined by spotting 0.2 µl of its solutions on a TLC plates, developing the plate with mobile phase M_{14} and visualizing the spots with ninhydrin. This process was repeated with successive reduction of the amount of lysine until no spot was detected. The minimum amount of lysine just visible on the TLC plate was taken as the limit of detection.

2.2.9 *Interference*

To examine the effect of the presence of cationic, anionic, and amino compounds (amines) as impurities on the separation of lysine, a solution of the foreign substance (cations, anions or amine; 0.2 µl) was spotted on silica layer followed by spotting of the mixture (0.2 µl) of the amino acids. After drying of the spot, TLC was performed with mobile phase M_{14}. Chromatography was performed as described above and the R_F values of lysine and the mixture of other amino acids were calculated after visualization with ninhydrin.

2.2.10 *Ageing effect of mobile phase*

To examine the ageing effect of the mobile phase M_{14} on the separation, the samples were spotted on the activated TLC plates, developed with freshly prepared mobile phase (M_{14}), and R_F values were calculated. The same process was repeated using the previously prepared mobile phase M_{14} at different intervals of time for 24 hours using the same batch of mobile phase M_{14}, and the R_F values were then calculated and compared.

2.3 RESULTS AND DISCUSSION

The purpose of this investigation was to develop a simple TLC method for specific separation of lysine from other essential amino acids. With this attention all the amino acids (Lys, Val, Ile, Thr, Met, Leu, Phe, and Trp) were chromatographed on silica gel layers using seventeen mobile phases, and optimum experimental

conditions were established for identification of lysine in the presence of the other acids in the pharmaceutical formulations Astymim (Forte) and Alamine (Forte) after preliminary separation from other components. The proposed method is important, because lysine is a useful constituent of dietary supplements. On the basis of the results obtained the possibilities of separation of lysine from a mixture with the seven other acids were examined. The separation patterns are shown in the figures. From these results several trends are noticeable.

(i) Lysine is barely separated from the other amino acids by use of DMW as mobile phase (**Figure 2.1 A**).The separation is also impossible with pure acetone as mobile phase, because all the amino acid, including lysine, reside at the point of application (**Figure 2.1 B**). Surprisingly, acetone-water mixtures (1:1 and 5:1,v/v) are capable of separating lysine from the other amino acids (**Figures 2.1 C - D**)

(ii) Aqueous solutions of a single surfactant (1.0×10^{-5} M TX-100 and 8.1×10^{-4} M SDS) or a mixture of surfactants (1.0×10^{-5} M TX-100 plus 8.1×10^{-4} M SDS) are not very effective and lead to poor separation of lysine (**Figure 2.2 A - C**)

(iii) Aqueous surfactant solutions with added acetone (mobile phase M_7 -M_{17}) result in better chromatographic performance, increasing the resolution of two adjacent spots. Compared with single-surfactant–acetone mobile phases (**Figures 2.3 A - B**), mixed- surfactant – acetone mobile phases (**Figure 2.4**) enable better separation. This observation is in agreement with our earlier findings- mixed surfactants were found to enable separation of aromatic amines [37]. The better chromatographic performance of the mixed surfactant solution may be attributed to specific interactions providing an altered microenvironment for separation. It is interesting to note from figures **(Figures 2.4 A - D)** that the chromatographic performance of aqueous mixed- surfactant systems was

found to depend upon amount of added acetone. It is clear from (**Figures 2.4)** that increasing the volume of acetone in the mobile phase results in increased resolution. An ion-pair mechanism probably operates during the separation process.

(iv) The excellent separation of lysine achieved in the presence of acetone (**Figure 2.5 A**) could not be achieved with aqueous mixed surfactants solution containing butan-2-one (**Figure 2.5 B**)

The results presented in **Table 2.1** clearly indicate that cations, anions, and amines do not affect the separation of lysine and it was always possible to isolate it from other acids.

It was also observed that mobile phase, M_{14} containing mixed surfactants with added acetone can be used continuously for 24 hour without any change in the R_F of lysine separated from its mixture with other amino acids. This shows the mobile phase is stable, without significant interactions among its components. The smallest possible amount of lysine visually detectable was 0.5μg per zone on silica TLC plates developed with M_{14}.

This study clearly reveals the better chromatographic performance of surfactant-mediated mobile phases in the presence of organic modifiers (acetone). This observation is in agreement with those from our previous study [13], in which acetone was identified as the most effective additive at 10% concentration with 3% Brij for separation of heavy metal cations on cellulose layers. Organic modifiers have been found to improve separation efficiency by reducing adsorption of the surfactant on stationary phase and altering the polarity of the mobile phase.

2.4 CONCLUSION

A new mobile phase system containing mixed aqueous surfactants Triton X-100 plus sodium dodecyl sulphate) and acetone has been established for specific

separation of lysine on silica gel layers. The proposed method can be used to identify the presence of lysine in pharmaceutical capsules.

2.5 APPLICATION

The practical utility of this method was examined by analysis of two commercially available pharmaceutical products Astymin and Alamine (both from Forte). It was concluded the method is suitable for identification of lysine in pharmaceutical formulations after preliminary separation from the other components (**Figures 2.6A - B**)

REFERENCES

[1] A. Mohammad, S. Hena, S. A. Bhawani, *Indian J. Chem.* **45** (2006) 1663.

[2] S. Khawas, S. Laskar, *J. Planar Chromatogr.- Mod. TLC* **16** (2003) 163.

[3] A. Mohammad, S. Hena, *Chromatography (Japan)* **25** (2004) 111.

[4] S. A. Nabi, M. A. Khan, *Acta Chromatographica* **13** (2003) 161.

[5] A. J. Mota, E. Castellanos, E. Juaristi, *Org. Prep. Proc. Int.* **35** (2003) 414.

[6] S. Khawas, S. Laskar, *J. Planar Chromatogr.-Mod TLC* **16** (2003) 163.

[7] A. Patrut, C. Marutoiu, O. F. Marutoiu, M. I. Moise, *J. Planar Chromatogr. –Mod TLC* **13** (2002) 151.

[8] A. Mohammad, V. Agrawal, *Indian J. Chem.* **40** (2001) 1130.

[9] L. Lepri, V. Coas, P. G. Desideri, *J. Planar Chromatogr.* **5** (1992) 175.

[10] R. Bhushan, J. Martins, Amino acids and their derivatives in Handbook of thin layer chromatography, 3rd ed J. Sherma and B. Fried (eds.), Marcel Dekker, Inc., 2003, 373.

[11] D. W. Armstrong, R. Q. Terrill, *Anal. Chem.* **51** (1979) 2160.

[12] M. G. Khaledi, *Trends Anal. Chem.* 7 (1988) 293.

[13] A. Mohammad, E. Iraqi, I. A. Khan, *J. Surfact. Deterg.* **2** (1999) 523.

[14] T. Okada, *J. Chromatogr. A.* **780** (1997) 343.

[15] G. M. Janini, H. J. Issaq, *J. Liq. Chromatogr. Related Technol.* **15** (1992) 927.

[16] M. F. Borgerding, R. L. Williams, W. L. Hinze, F. H. Quina, *J. Liq. Chromatogr.* **12** (1989) 1367.

[17] Y. Moroi, H. Akisada, M. Saito, R. Matvura, *J. Colloid Interface Sci.* **61** (1977) 233.

[18] K. Saito, *Kagaku,* **45** (1990) 884.

[19] M. Almgre, S. Swarup, *J. Colloid Interface Sci.* **91** (1983) 256.

[20] A. S. Kord, M. G. Khaledi, *Anal. Chem.* **64** (1992) 1901.

[21] A. Berthod, I. Girad, C. Gonnet, *Anal. Chem.* **58** (1986) 1359.

[22] A. Berthod, X. Jun, A. Serge, C. Gonnet – Colette, Can. *J. Chem.* **74** (1996) 227.

[23] M. M. Bushey, J. W. Jorgenson, *Anal. Chem.* **61** (1989) 491.

[24] J. G. Dorsey, M. T. De Echegarary, J. S. Landy, *Anal. Chem.* **55** (1983) 924.

[25] S. N. Shtykov, E. G. Sumina, *J. Anal. Chem. (Russian)* **53** (1998) 446.

[26] A. Mohammad, S. Anwar, V. Agrawal, *J. Indian Chem. Soc.* **76** (1999) 452.

[27] S. N. Shtykov, E. G. Sumina, N. V. C. Tyurina, *J. Planar Chromatogr.-Mod TLC* **13** (2000) 266.

[28] A. Mohammad, V. Agrawal, *J. Planar Chromatogr. –Mod TLC* **13** (2000) 210.

[29] M. A. Hernandez-Torres, J. S. Landy, J. G. Dorsey, *Anal. Chem.* **58** (1986) 744.

[30] A. Mohammad, N. Jabeen, *Indian J. Chem. Technol.* **10** (2003) 79.

[31] K. K. Gosh, A. Pandey, *J. Indian Chem. Soc.* **76** (1999) 191.

[32] A. Shiloach, D. Blankschtein, *Langmuir* **14** (1998) 1618.

[33] S. De, V. K. Aswal, P. S. Goyal, S. Bhattacharya, *J. Phys. Chem.*(B) **101** (1997) 5639.

[34] M. N. Khan, Micellar Catalysis,Taylor and Francis, Boca Raton, FL, 2007.

[35] S. Morisada, H. Shinto, K. Higgashitani, *J. Phys. Chem.* (B) **109** (2005) 11762.

[36] P. M. Claesson, M. Kjellin, O. J. Rojas, C. Stubenrauch, *J. Phy. Chem.* **8** (2006) 5501.

[37] A. Mohammad, I. A. Khan, *Indian J. Chem. Technol.* **8** (2001) 244.

Table 2.1: Separation of lysine from a mixture of essential amino acids (Mx) in the presence of cations, anions and amines. The stationary phase was silica gel G and the mobile phase M_{14}

Impurities	R_F of lysine	R_F of Mx
Cations		
Hg^{2+}, Pb^{2+},Cu^{2+}, Th^{+4}, Zn^{2+}, Fe^{3+}, Cd^{2+}, Cr^{6+}, Ni^{2+}, Co^{2+},Mo^{2+}, Al^{3+} ,Tl^{3}	0.08 -0 .18	0.80 –0.90
Anions		
Cl^-, SO^{2-}_4, NO_3^-, CN^-, $PO^{3}_4^-$,I^-, ClO_4^-	0.05 - 0.16	0.80 - 0.95
Amines		
Naphthylamine, methylamine, diphenylamine, tri-n-butylamine, tert.butylamine and m-phenylenediamine	0.05-0.19	0.80 –0.95

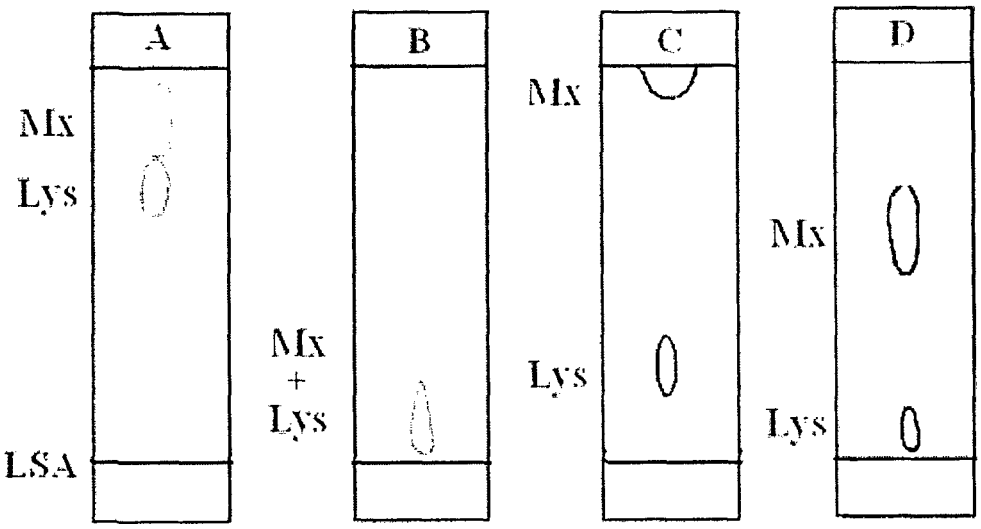


Figure 2.1: Chromatograms showing the separation of lysine from other amino acids on silica gel layers developed with aqueous mobile phase systems containing acetone

A Double distilled water (DDW)

B Acetone

C DDW plus acetone (1:1, v/v)

D DDW plus acetone (1:5, v/v)

Mx = Mixture of seven essential amino acids

Lys = L-Lysine

LSA= Line of sample application

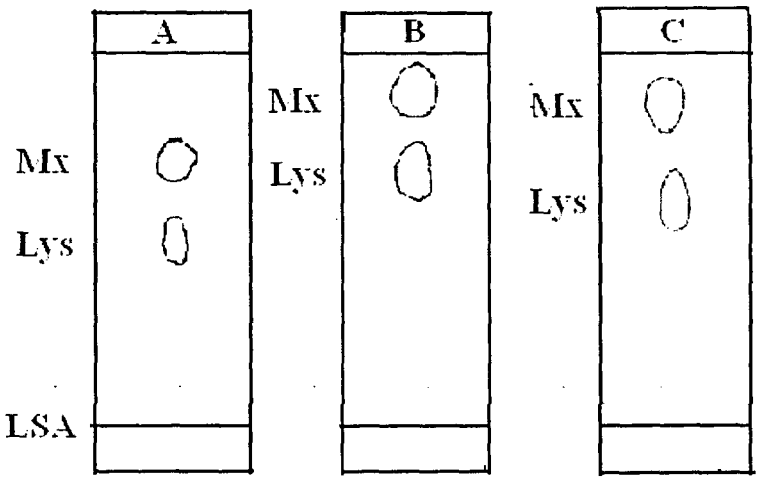


Figure 2.2: Chromatogram showing the separation of lysine from other amino acids on silica gel layers developed with aqueous solutions of single and mixed surfactants as mobile phases

A 1.0×10^{-5} **M TX-100**

B 8.1×10^{-4} **M SDS**

C 1.0×10^{-5} **M TX-100 plus** 8.1×10^{-4} **M SDS (1:1, v/v)**

Other abbreviated as for Figure 2.1

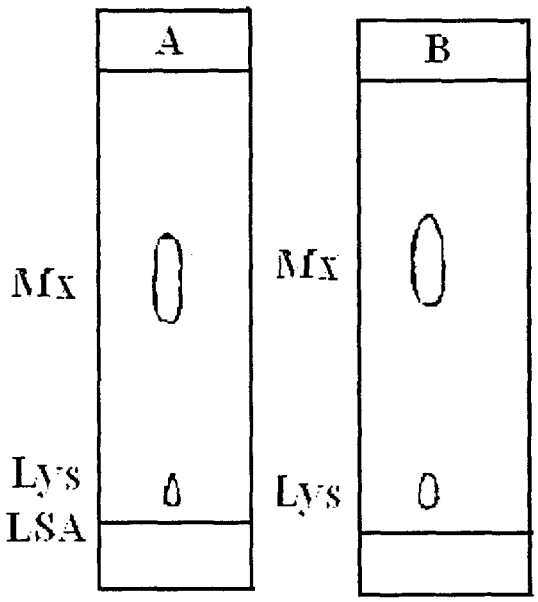


Figure 2.3: Chromatogram showing the separation of lysine other amino acids on silica gel layers developed with aqueous solution of a single surfactant containing acetone as mobile phase

A 8.1×10^{-4} **M SDS plus acetone (1: 5, v/v)**

B 1.0×10^{-5} **M TX-100 plus acetone (1: 5, v/v)**

Other abbreviated as for Figure 2.1

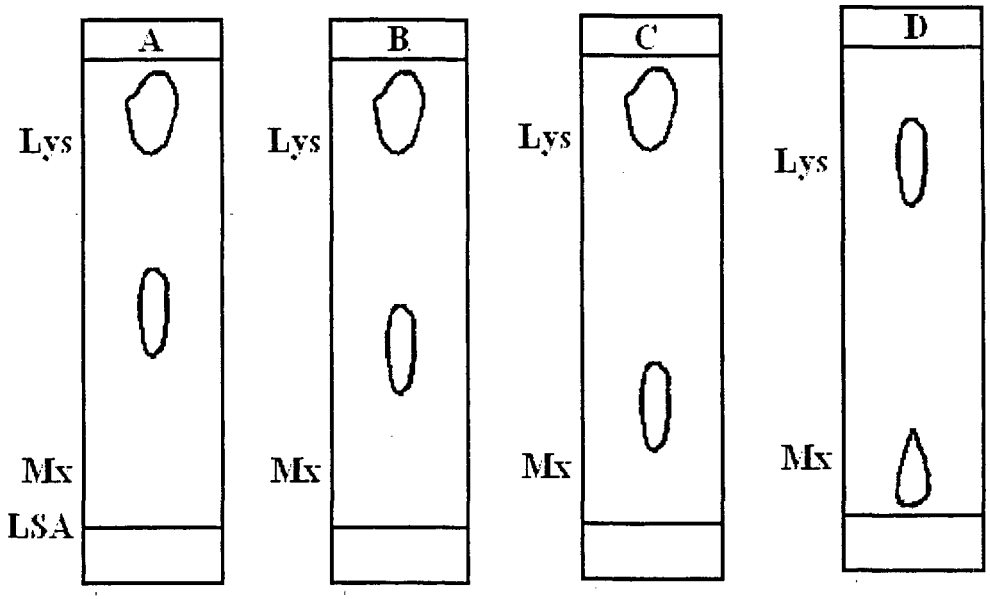


Figure 2.4: Chromatogram showing the separation of lysine from other amino acids on silica gel layers developed with surfactant- containing mobile phase

A 1.0×10^{-5} **M TX-100 plus 8.1** $\times 10^{-4}$ **M SDS plus acetone (1:1:1, v/v/v)**

B 1.0×10^{-5} **M TX-100 plus 8.1** $\times 10^{-4}$ **M SDS plus acetone (1:1:2, v/v/v)**

C 1.0×10^{-5} **M TX-100 plus 8.1** $\times 10^{-4}$ **M SDS plus acetone (1:1:3, v/v/v)**

D 1.0×10^{-5} **M TX-100 plus 8.1** $\times 10^{-4}$ **M SDS plus acetone (1:1:5, v/v/v)**

Other abbreviated as for Figure 2.1

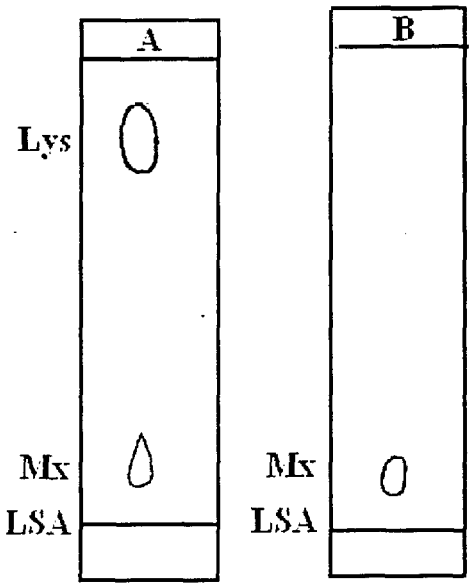


Figure 2.5: Comparison of chromatograms showing the separation efficiency of mobile phase containing acetone and butan-2-one on silica gel layers

A 1.0×10^{-5} **M TX-100 plus** 8.1×10^{-4} **M SDS plus acetone (1:1:5, v/v/v)**

B 1.0×10^{-5} **M TX-100 plus** 8.1×10^{-4} **M SDS plus butan-2-one (1:1:5, v/v/v)**

Other abbreviated as for Figure 2.1

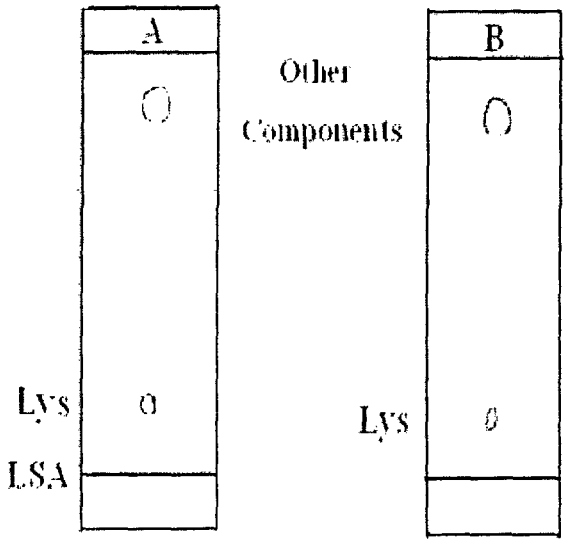


Figure 2.6: Chromatogram showing the separation on silica gel layers developed with 1.0 x 10^{-5} M TX-100 + 8.1 x 10^{-4} M SDS + acetone (1:1:5, v/v/v) of lysine from other component

A Astymine (Forte) and

B Alamine (Forte)

Other abbreviated as for Figure 2.1

CHAPTER-3

Identification and Simultaneous Separation of Six Hydrophilic Therapeutic Vitamins by Micellar Thin Layer Chromatography

3.1 INTRODUCTION

Thin Layer Chromatography (TLC) represents one of the most important analytical techniques for identification and simultaneous separation of organic compounds [1-4]. Vitamins, the amphipathic molecules are the precursors of various metabolic pathways going in every living organism. These organic molecules essentially play important functions as coenzymes and antioxidants. They occur in almost all dietary products. Their deficiency may lead to numerous disorders. To overcome their loss in pathological conditions, they are available in the form of various therapeutic multivitamin dosages, which are composed of B-group vitamins, folic acid, ascorbic acid, biotin, calcium pantothenate fortified with minerals and antioxidants.

According to literature, various methods like HPLC using diode array detector in commercial multi-vitamin preparation [5], HPLC [6], reverse phase-HPLC (RP-HPLC) [7], TLC using fibre–optic fluorometric quantitation [8] and quantitative determination by fluorescence quenching [9,10], HPTLC [11] and RP-TLC [12] have been used in the analysis of vitamins. Most of thin layer chromatographic procedures developed for their analysis involve the use of organic or mixed aqueous organic mobile phases [13]. Since the first report [14] on the use of aqueous solution of surfactant as mobile phase, several workers [15] have utilized it in the analysis of closely related organic as well as inorganic substances by Ion–Pair and Micellar TLC [16-18].

The excellent chromatographic performance of surfactant-mediated systems in the presence of small amounts of added organic solvents has generated renewed interest in chromatography [19-21]. The favorable features of surfactants as modifiers of mobile and stationary phases in TLC have been admirably documented by Sumina *et al.* in recent review [22].

The most interesting feature of surfactant solutions is their dual hydrophobic and hydrophilic nature that provides electrostatic as well as hydrophobic interactions leading to unusual separation possibilities [23-25]. The capability of simultaneous

separation of ionic and non-ionic compounds is another fascinating feature of micellar systems.

Encouraged by the above mentioned advantageous features of micellar solutions systems, a new TLC system consisting of silica gel 60 F_{254}HPTLC plates in combination with aqueous micellar Sodium dodecyl sulphate (SDS) an anionic surfactant containing acetonitrile as organic modifier has been identified as the most favorable for identification and separation of B-group vitamins along with folic and ascorbic acids under UV radiations (λ=254 nm). SDS was selected because of the fact that amongst micelles of surfactants [26,27] found most efficient especially with silica gel.

The present study is aimed to develop a reliable and efficient micellar TLC method for simultaneous separation and detection of B_1, B_2, B_6, B_{12}, ascorbic acid and folic acid .The proposed method has been applied to the analysis of multivitamin formulation (Becosules* capsules).

3.2 EXPERIMENTAL

All experiments were performed at 30 ± 2 °C

3.2.1 *Apparatus*

High Performance Thin Layer Chromatography (HPTLC) aluminum sheet of silica gel 60 $_{F254}$ plates B.N 1.05554 from Merck Germany and TLC twin-trough chamber Camag, Muttenz, Switzerland lined with lid were used.

3.2.2 *Reagents*

Cetyltrimethyl ammonium bromide (CTAB) and Triton X-100 (Iso-octylphenoxy polyethoxyethanol) were procured from CDH India. Acetonilrile (Lichrosolv) from

Merk, India. Sodium dodecyl sulphate (SDS) was purchased from BDH India. All reagents were of Analytical grade.

3.2.3 *Chemical solutions*

Aqueous solutions of TX-100, SDS and CTAB were prepared in DDW. Standard test solutions of vitamin B_2 (0.01% w/v), folic acid (0.1% w/v), B_{12} (0.2% w/v), ascorbic acid and vitamin B_1 (1.0% w/v) and of vitamin B_6 (2.0% w/v) were also prepared in double distilled water. For sample preparation, standard test solutions (0.3 ml) of above listed vitamins were mixed and 0.50µl of this mixture was used for chromatography. **Table 3.1** denotes the chemical names of some water soluble vitamins.

3.2.4 *Dosage formulation*

Becasules* capsules (B-complex forte with vitamin C) Pfizer, India, analysed by the proposed method has the following composition (mg):

1) Thiamine mononitrate IP	10
2) Riboflavin IP	10
3) Pyridoxine hydrochloride IP	03
4) Vitamin B_{12} IP (as Stablets 1:100)	15
5) Niacinamide IP	100
6) Calcium pantothenate	50
7) Folic acid IP	1.5
8) Biotin USP	100
9) Ascorbic acid IP	150

3.2.5 *Preparation of dosage solutions / sample preparation*

Becasules *capsule was finely powdered and dissolved in 10 ml double distilled water (S_1). The resultant solution was spiked with 0.2 ml of cyanocobalamine (0.2%) and 0.2 ml of folic acid (0.1%). This mixture was treated as commercial analyte sample. For studying nature of sample solvent, several analyte samples were prepared by dissolving the powder of Becosules*capsule separately in 10ml of ethanol (S_1), 0.04M orthophosphoric acid (S_2), 0.24M sodium hydroxide (S_3), 0.001M SDS (S_4) and 0.001M CTAB (S_5).

3.2.6 *Detection*

UV radiation (λ=254 nm) was used to detect all the studied vitamins except B_{12} which was observed visually in sunlight as pink spot. The plates were photographed under 254 nm using Nikon cool pix 6.2 megapixel digital camera.

3.2.7 *Stationary phase*

Silica gel 60 F_{254} high performance thin layer chromatographic plates.

3.2.8 *Mobile phases*

The solvents systems used as mobile phases are listed below

Symbol	Composition
M_1	Acetonitrile
M_2	Aqueous SDS solution (4%)
M_3	Aqueous SDS solution 4% + acetonitrile (1:1, v/v)
M_4	Aqueous SDS solution 4% + acetonitrile (1:2, v/v)
M_5	Aqueous SDS solution 4% + acetonitrile (1:3, v/v)
M_6	Aqueous SDS solution 2% + acetonitrile (1:2, v/v)
M_7	Aqueous SDS solution 4% + acetonitrile (2:1, v/v)
M_8	Aqueous SDS solution 6% + acetonitrile (1:2, v/v)
M_9	Aqueous TX-100 solution 4% + acetonitrile (1:2, v/v)
M_{10}	Aqueous CTAB solution 4% + acetonitrile (1:2, v/v)

3.2.9 *Procedure*

Silica gel 60 F_{254} HPTLC plates were activated at 60 ± 2 °C in an electrically controlled oven for 20 minutes and stored in closed chamber until used. Spotting of 0.50 µl of sample using micropipette (Tripette 0783178, Germany) was done at 1.0 cm from the base of HPTLC plates. Spots were dried in air and developed in a closed presaturated glass chamber with desired mobile phase by ascending technique up to the ascent of 5 cm from the point of application. After development, the HPTLC plates were withdrawn from glass chamber, air dried and then detected under UV light.

3.3 RESULTS AND DISCUSSION

Chromatography of six hydrophilic vitamins was performed using various hybrid mobile phases composed of 4% aqueous SDS and acetonitrile in different volume ratio. The mobility pattern of vitamins, viewed under UV radiations (λ=254 nm) was found to depend on the composition of mobile phase. The results obtained during entire study have been summarized in **Figures (3.1-3.12)**.

From the results shown in Figures following trends are noticeable.

(i) With acetonitrile (M_1) as mobile phase, all vitamins remain at the point of application as highly compact and well formed spots (**Figure 3.1**)

(ii) With SDS micellar solution (M_2), all vitamins show significant mobility producing diffused spots, which appeared near the solvent front. The cyanocobalamine was the exception, which appeared at the middle of TLC plate. None of the vitamins produces compact spot (**Figure 3.2**).

(iii) As expected, the hybrid mobile phase systems (M_3- M_5) prepared by mixing 4% aqueous solution of SDS and acetonitrile in different volume ratios gave useful results, which can be utilized in chromatographic analysis of vitamins (**Figure 3.3 - 3.5**). In general, on the increase or decrease of SDS volume in the mobile phases M_6 and M_8, all vitamins tended to smear along the length

of the chromatogram rather than move as discrete spot (**Figure 3.6**). Out of these mobile phases, system constituting of 4% aqueous SDS + acetonitrile (1:2, v/v) was found most favorable for the separation and identification of vitamins from their mixture in the sample. The substitution of SDS in M_4 by 4% aqueous TX-100 (a nonionic surfactant) or 4% aqueous CTAB (a cationic surfactant) could not produce useful results (**Figure 3.7, 3.8**) and hence the TLC system comprising of HPTLC silica as stationary phase and 4% aqueous SDS plus acetonitrile (1:2, v/v) termed as M_4 in this paper was selected for further study (**Figure 3.9**).

(iv) The selected TLC system was tested for its applicability on marketed formulation (Becasules* capsule) as follows:

(a) For identification of vitamins on HPTLC plate, the capsule sample dissolved in double distilled water (S_1) was chromatographed on HPTLC plate and the pattern realized for identification of vitamins is shown in (**Figure 3.10**). The effect of few essential amino acids on separation pattern of vitamins was also examined and the results are presented in (**Figure 3.11**) .The presence of amino acids does not influence the separation and identification of vitamins.

(b) In order to understand the effect of sample solvent on chromatographic performance of vitamins, the solution of sample (Becasules *capsule) was prepared in S_2, S_3, S_4 and S_5 separately. The chromatographic behavior of the resultant solution of vitamins is shown in (**Figure 3.12**). CTAB is the only substance to influence the separation of vitamins. Thus, vitamins can be analyzed in a commercial sample under different solvent conditions. All the samples were found stable for the duration of 48 hours as the monitoring of sample at different intervals revealed almost identical chromatogram. It is evident from figure that the chromatographic patterns of standard sample solution (**Figure 3.9**) and commercial sample solution (**Figure 3.10**) are not identical. The difference may be attributed to the presence of other components (biotin, calcium pantothenate and nicotinamide) in the

commercial sample.

(v) The lowest possible detectable amount of vitamins B_1, B_2, B_6 and B_{12}, folic acid and L-ascorbic acid on silica $60F_{254}$ HPTLC plates developed with M_4 ranges from 0.002-0.400µl. Thus, the proposed method is highly sensitive for on-plate detection of vitamins. The limit of detection of vitamins is listed in **Table 3.2.**

3.4 CONCLUSION

A new micellar mobile phase comprising of 4% aqueous SDS + acetonitrile (1:2 v/v) is developed for rapid analysis of therapeutic vitamins. Being selective, the proposed TLC method could be easily implemented as a reliable analytical tool for the separation and identification of hydrophilic vitamins in pharmaceutical formulations.

REFERENCES

[1] H. Wagner, S. Bladt, (eds) *Plant Drug Analysis. A Thin Layer Chromatography* Atlas, Springer, Berlin, Germany (1995).

[2] J. Sherma, B. Fried (eds) Handbook of Thin Layer Chromatography, (3rd ed), **89** (2003), Marcel Dekkar, inc: New york.

[3] C. Cimpoiu, A. Hosu, *J. Liq. Chromatogr. Related Technol.* **30** (2007) 701.

[4] A. Mohammad, S. Laeeq, *J. Planar. Chromatogr.- Mod. TLC* **20** (2007) 65.

[5] K-On Chu, K. C. Tin, *Anal. Lett.* **31** (1998) 2707.

[6] X. Huang, I. Gusev, C. Horvath, *J. Chromatogr. A* **855** (1999) 273.

[7] R. Bhushan, A. Meenakshi, *Natl. Acad. Sci. Letters.* **25** (2002) 159.

[8] D. A. Navas, P. A. Guirado, S. F. Garcia, *J. Chromatogr.* **655** (1993), 39.

[9] B. Srinivas, K. Srinivasulu, Vikram, *J. Indian Chem. Soc.* **69** (1992) 669.

[10] N. U. Perisic Janjic, M. R. Poponic, T. J. Djakovic, *Acta Chromatogr.* **5** (1995) 144.

[11] L. A. Kartsova, O. A. Koroleva, *J. Anal. chem.* **62** (2007) 255.

[12] I. Baranowska, A. Kadziolka, *Acta Chromatog.* **6** (1996) 61.

[13] F. Watanabe, E. Miyamoto, *Chromatogr. Sci. Ser. (Eng).* **95** (2006) 237.

[14] D. W. Armstrong, R. Q .Terrill, *Anal. Chem.* **51** (1997) 2160.

[15] J. G. Dorsey, M. T. DeEchegaray, J. S. Landy, *Anal. Chem.* **55** (1983) 924.

[16] A. S. Kord, M. G. Khalidi, *Anal. Chem.* **64** (1992) 1901.

[17] A. Mohammad, N. Jabeen, *Indian J. Chem. Technol.* **10** (2003)79.

[18] A. Mohammad V. Agrawal , S .Hena, *Adsorpt. Sci. Technol.* **22** (2004) 89.

[19] M. G. Khaledi, *Anal. Chem.* **7** (1988) 293.

[20] M. F. Borgerding, R. L. Williams, W. L. Hinze, F. H. Quina, *J. Liq. Chromatogr.* **12** (1989) 1367.

[21] S. N. Shtykov, E. G. Sumina, E. V. Smushkina, N. V. Tyurina, *J. Planar Chromatogr* . **12** (1999) 129.

[22] E. G. Sumina, S. N. Shtykov, N. V. Tyurina, *J. Anal. Chem.* 58 (2003) 720.

[23] M. A. Hernandez – Torres, J. S. Landy, J. G. Dorsey, *Anal. Chem.* **58** (1986) 744.

[24] A. Berthod, M. De Carvalho, *Anal. Chem.* **64** (1992) 2267.

[25] M. Almgren, S. Swarup, *J. Colloid Interface Sci.* **91** (1983) 256.

[26] A. Mohammad, Y. H. Sirwal, *J. Anal. Chem.* **58** (2003) 731.

[27] A. Mohammad, E. Iraqi, I. A. Khan, *J. Surfactants Deterg.* **2** (1999) 523.

Table 3.1: List of some water - soluble vitamins studied

Vitamin	Common name	Chemical name
B_1	Thiamine hydrochloride. (CDH, India)	(3-{(4-amino-2-methyl-5-pyrimidinyl) methyl]-5-(2-hydroxyethyl)-4-methyl thiazolium chloride hydrochloride.
B_2	Riboflavin (CDH, India)	3,10-dihydro-7,8-dimethyl-10-[(2S,3S,4R)-2,3,4,5-tetrahydroxypentyl]benzo-[g]pteridine-2,4-dione7,8-dimethyl-10-(1'-D-ribityl)isoalloxazine.
B_6	Pyridoxine hydrochloride (BDH, India)	(hydroxy-6-methyl-3,4-pyridine dimethanol hydrochloride.
B_{12}	Cyanocobalamine (CDH, India)	α-(5,6-dimethylbenzimidazolyl cobamidcyanide.
C	L-ascorbic acid (CDH, India)	2, 3-endiol-L-gulonicacid-γ-lactone.
Folic acid	Folic acid (CDH, India)	(N-{4-{{2-amino-1,4-dihydro-oxo-6 pteridinyl)methyl}amino}benzoyl]-L-glutamic acid.

Table 3.2: Limit of detection of water-soluble vitamins

Vitamin	Limit of detection (μg)
B_1	0.200
B_2	0.002
B_6	0.400
B_{12}	0.040
C	0.200
Folic acid	0.020

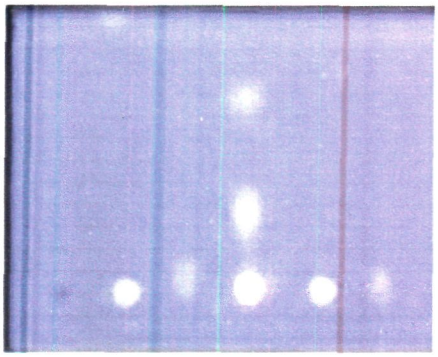


Figure 3.1: Chromatogram showing the mobility pattern of vitamins B_{12}, B_1, B_6, B_2, folic acid and vitamin C from left to right, plate developed with mobile phase M_1

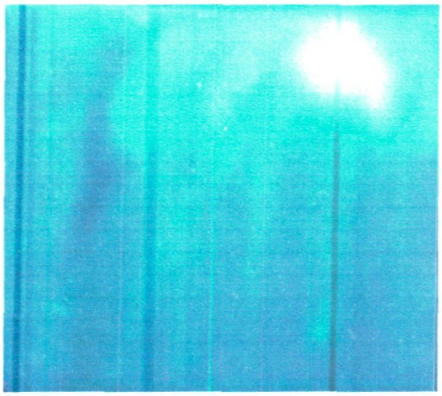


Figure 3.2: Chromatogram showing the mobility pattern of vitamins B_{12}, B_1, B_6, B_2, folic acid and vitamin C from left to right, plate developed with mobile phase M_2

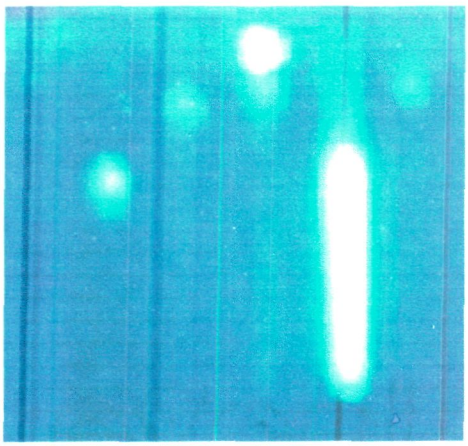


Figure 3.3: Chromatogram showing the mobility pattern of vitamins B_{12}, B_1, B_6, B_2, folic acid and vitamin C from left to right, plate developed with mobile phase M_3

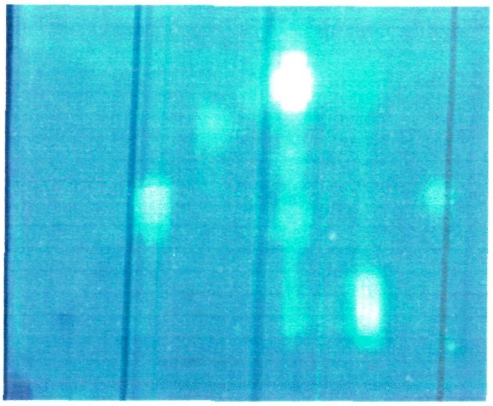


Figure 3.4: Chromatogram showing the mobility pattern of vitamins B_{12}, B_1, B_6, B_2, folic acid and vitamin C from left to right, plate developed with mobile phase M_4

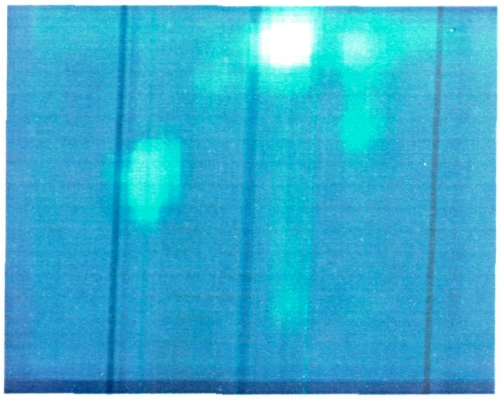


Figure 3.5: Chromatogram showing the mobility pattern of vitamins B_{12}, B_1, B_6, B_2, folic acid and vitamin C from left to right plate developed with mobile phase M_5

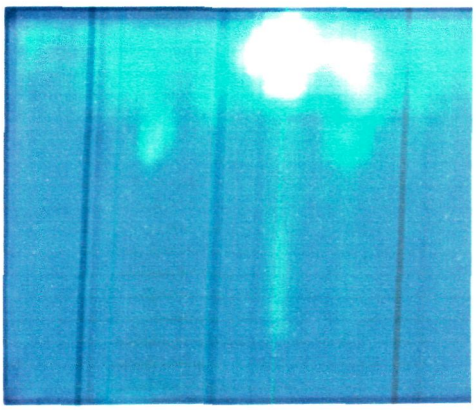


Figure 3.6: Chromatogram showing the mobility pattern of vitamins B_{12}, B_1, B_6, B_2, folic acid and vitamin C from left to right, plate developed with mobile phase M_6

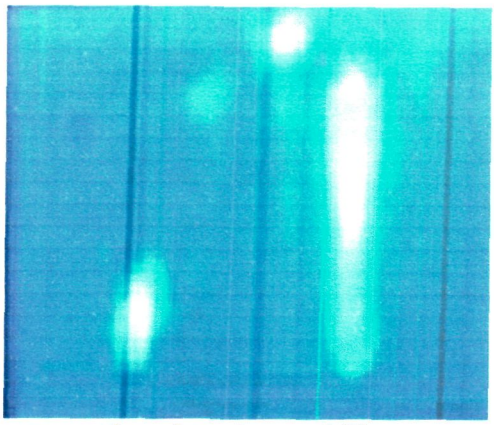


Figure 3.7: Chromatogram showing the mobility pattern of vitamins B_{12}, B_1, B_6, B_2, folic acid and vitamin C from left to right, plate developed with mobile phase M_9

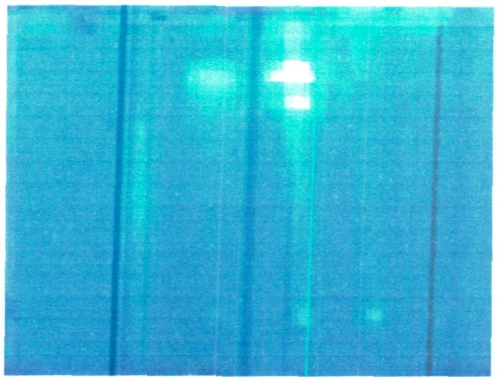


Figure 3.8: Chromatogram showing the mobility pattern of vitamins B_{12}, B_1, B_6, B_2, folic aand C from left to right, plate developed with mobile phase M_{10}

Figure 3.9: Chromatogram showing the mobility pattern of vitamins B_{12}, B_1, B_6, B_2, folic caid and vitamin C from their mixture (standard sample), plate developed with mobile phase M_4

Figure 3.10: Chromatogram showing the resolution of vitamins present in Becasules Capsule, plate developed with mobile phase M_4

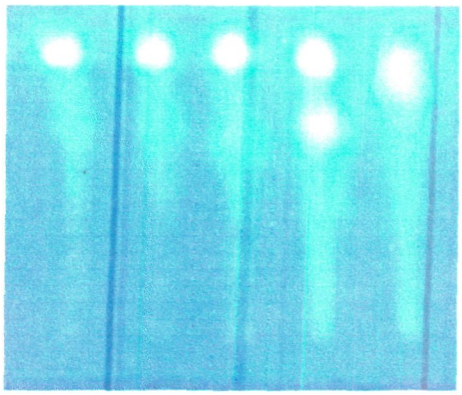


Figure 3.11: Effect of essential amino acids as impurity added in Becasules Capsules, plate developed with mobile phase M_4

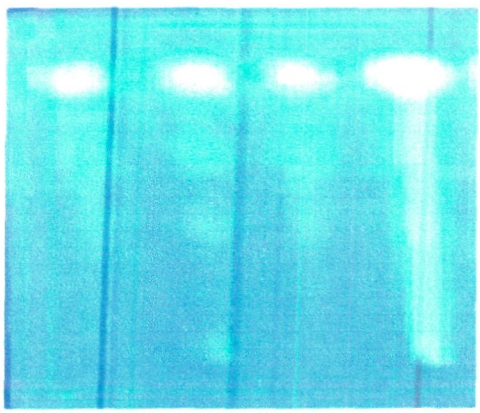


Figure 3.12: Effect of sample solvents S_2, S_3, S_4 and S_5 from left to right for chromatographic performance of Becasules Capsule, plate developed with mobile phase M_4

CHAPTER-4

Identification of Coexisting Pentose, Hexose and Disaccharides with Preliminary Separation through Hydrophilic Interaction on Silica HPTLC Plate Using Aqueous Sodium Deoxycholate-Acetonitrile Mobile Phase System

4.1 INTRODUCTION

Among the natural substances, carbohydrates are most widely distributed with diverse roles and hence the analysis of carbohydrates in food, beverages and pharmaceutical formulations has been an attractive area of research for chromatographers [1-3]. As regards to the use of thin layer chromatography(TLC), in most cases, mixture of organic solvents and mixed aqueous-organic eluents comprising of chloroform, pyridine, benzene etc. as one of the components have been used for the analysis of polar sugars [4,5]. Mixtures of acetonitrile with water as eluent in combination with silica gel or amino-bonded layers have been, generally, preferred to resolve complex mixtures of sugars on TLC plates [6,7].As an alternative to mixed aqueous-organic eluents, use of micellar bile salt mobile phases in chromatographic separation of isomeric compounds has been reported by R. W. Williams *et al.* [8]. This study opened a novel route for replacing conventional hydro-organic mobile phase systems by bile salt surfactant solutions with judicious use of organic additives. It has generated the renewed interest of chromatographers in the use of bile salt as mobile phase or in the stationary phase to achieve the separations of analytical importance [9-11].

Bile salts being naturally occurring steroid-based surfactants play an important role in biological systems and have been a subject of numerous studies [12,13]. Sodium salt of cholic acid form helical aggregates in aqueous solutions which has lateral surface with backbone of steroid having angular methyl outside and hydroxyl inside groups the helix. More recently, the use of bile salt instead of long-chain surfactants for separating a range of neutral analytes as well as for chiral separations has been reported [14]. Surprisingly, salts of bile acids have not been used in TLC or high performance (HP) TLC for the analysis of sugars whereas limited use of traditional linear surfactants (sodium dodecyl sulphate) has been reported [15]. Thus, it is worthwhile to utilize the analytical potential of physiologically important surfactant in chromatographic analysis of biomolecules. Micellar mobile phases are expected to be very effective in the resolution of disaccharide from monosaccharide.

Hydrophilic interaction chromatography (HILIC) is a variant of normal phase chromatography which is specially useful for the separation of highly polar substances including biologically active compounds, such as pharmaceutical drugs, neurotransmitters, nucleosides, nucleotides, amino acids, peptides, proteins, oligosaccharides, carbohydrates, etc [16]. The mechanism of separation is liquid-liquid partition that is by passing a hydrophobic or mostly organic mobile phase across a neutral hydrophilic stationary phase (bare silica) causing solutes to elute in the order of increasing hydrophilicity [17]. The retention of polar analytes often requires a highly aqueous mobile phase to achieve retention which sometimes lead to problems such as phase collapse (dewetting) [18].The water-deficient mobile phase and water-rich layer on the surface of the polar stationary phase creats a liquid/liquid extraction system. The analyte is distributed between these two layers. However, HILIC is not simply involves partitioning but also includes hydrogen donor interactions between neutral polar species as well as weak electrostatic mechanisms under the high organic solvent conditions used for retention.

A typical mobile phase for HILIC chromatography includes acetonitrile (ACN) with a small amount of water. Ionic additives, such as ammonium acetate and ammonium formate, buffers and salts like sodium percholate are usually used to control the mobile phase pH and ion strength. In HILIC they can also contribute to the polarity of the analyte, resulting in differential changes in retention. For the separation of neutral analytes like carbohydrates, the role of buffer is secondary.

For the first time an aqueous micellar solution of biosurfactant (sodium deoxycholate) is being in mobile phase containing bulk of acetonirtile for on-plate chromatographic resolution of multicomponent mixtures of neutral sugars. During the present study, aqueous micellar sodium deoxycholate (1%, w/v) plus acetonitrile (1:5, v/v) was found as the most optimum eluent for separation of disaccharides from pentoses and hexoses. The purpose of this communication is to explore the utility of biocompatible mobile phase systems for on-plate identification of sugars with preliminary separation on silica HPTLC plates.

4.2 EXPERIMENTAL

All experiments were performed at 30 ± 1°C

4.2.1 *Apparatus*

The chromatography was performed in 24 cm x 6 cm glass jars. A glass sprayer was used to spray reagent on the plates to locate the position of the spot of analyte.

4.2.2 *Reagents*

Sugars (glucose, C_1 and lactose monohydrate, C_7) were from Merck (India) whereas D-fructose, C_2; maltose monohydrate, C_8; D-galactose, C_3; D-mannose, C_4; D-ribose, C_5; and D (+) xylose, C_6; were procured from CDH, India. Acetonitrile, sodium dodecyl sulphate (SDS), and acetone (Merck,India), methanol (Qualigens,India), tween 20 (Tw-20) and cetylpryridium bromide, CPB (CDH, India) were used. All chemicals were of analytical grade.

Bile salts (sodium deoxycholate (NaDC), sodium cholate (NaC) and sodium taurocholate (NaTC) from Loba chemie were used. Their structures are shown in **Figure 4.1(a-c)**.

4.2.3 *Test solution*

Aqueous solutions (1% w/v) of glucose, lactose monohydrate, D-fructose, maltose monohydrate, D-galactose, D-mannose, D-ribose and D (+) xylose were prepared.

4.2.4 *Sample preparation*

Pharmaceutical formulations, Honitus (C_9) and Becozinc (C_{10}) (1% v/v) were prepared in double distilled water (DDW).

4.2.5 *Preparation of biological blood serum sample (C_{11} and C_{12})*

Since glucose is distributed almost uniformly between cells and plasma, either

whole blood or plasma or serum can be taken. Venous capillary blood is often convenient as multiple specimens may be taken from the same patient and an anti-glycolytic agent such as sodium fluoride should be added. Small stopped tubes are used containing a suitable anti-glycolytic preservative anticoagulant. Capillary tubes are maintained in a sealed condition until analyzed. It is required to take venous blood with the help of disposable syringes, the needle is removed and the blood sample is allowed to run into the sample bottle. The taken venous blood is run into a clean dry vessel and allowed to stand at room temperature until it has clotted. It should then be kept in a warm place until the clot has retracted and the serum separated. The blood must not be chilled as this causes heamolysis as well as abnormal plasma cell distribution of certain ions.

4.2.6 *Detector*

Sugars were detected by orcinol solution prepared by mixing 250 mg orcinol, 7.5 ml conc. sulphuric acid, 200 ml ethanol and 50 ml DDW. Color was developed on plate by spraying this solution and heating HPTLC plates at 110°C for 5-10 min in an electrically controlled oven. The detected spots appeared as brown- purple for all disaccharides and hexoses except D-fructose (appeared as orange) and blue for pentoses.

4.2.7 *Stationary phase*

High performance thin layer chromatographic (HPTLC) plates of silica gel 60 backed with aluminium from Merck, Germany were used as stationary phase for the entire study.

4.2.8 *Mobile phases*

Following mobile phases were used in the experiment

Code	Mobile phase composition (v/v)
M_1	DDW
M_2	1% NaDC
M_3	ACN
M_4	DDW : ACN (1:5)
M_5	NaDC : ACN (1:9)
M_6	NaDC : ACN (1:8)
M_7	NaDC : ACN (1:5)
M_8	NaDC : ACN (1:4)
M_9	NaDC : ACN (1:3)
M_{10}	NaDC : ACN (3:7)
M_{11}	NaDC : ACN (1:2)
M_{12}	NaDC : ACN (1:1)
M_{13}	NaDC : ACN (7:3)
M_{14}	NaDC: ACN (5:1)
M_{15}	NaDC : ACN (9:1)
M_{16}	NaC : ACN (1:5)
M_{17}	NaTC : ACN (1:5)
M_{18}	SDS : ACN (1:5)
M_{19}	Tw-20 : ACN (1:5)
M_{20}	CPC : ACN (1:5)
M_{21}	NaDC:MeOH (1:5)
M_{22}	NaDC : CH_3CHO (1:5)

4.2.9 *Procedure*

Silica gel HPTLC plates were activated at 60 ± 1 °C in an electrically controlled oven for 30 min and stored in closed chamber until used. Spotting of 0.20 µl of

sample using micropipette (Tripette, Germany) was done at 1.0 cm from the lower edge of HPTLC plates. Spots were air dried and developed in a closed pre-saturated glass chamber with desired mobile phases by ascending technique upto 5 cm from the point of application. After development (development time 5-8 min. depending upon the composition of mobile phase), the HPTLC plates were withdrawn from glass chamber, air dried and analytes were detected by spraying ethanolic orcinol solution by means of glass sprayer and heating at 110°C for 5-10 min. The R_F values were calculated as:

$$R_F = \frac{\text{Distance of solute migration from the origin}}{\text{Distance of solvent migration from the origin}}$$

The standard deviation in R_F values was also calculated using the formula

$$S.D = \sqrt{\sum (Xi-X)^2 / (n-1)}$$

Where Xi is the individual R_F value, X is the mean R_F value and (n-1) is the number of observations made for each sugar.

4.2.10 *Separation*

For separation of the three series (pentose, hexose and disaccharide), equal volumes of all sugar were mixed and 0.20 µl of the resultant mixture was applied on the activated silica gel HPTLC plates. The plates were developed with mobile phase M_7, the spots were detected and the hR_F (R_F x 100) values of the separated sugars were calculated.

4.2.11 *Interference*

The aqueous solutions (1% w/v) of metal ions (Tl^+, Cu^{2+}, Zn^{2+}, Ni^{2+}, Bi^{3+}, VO^{2+}, Th^{4+}, Hg^{2+}, Co^{2+}, CrO_4^{2-}, Al^{3+}, Pb^{2+}) prepared from their chloride, nitrate or sulphate salts, were used for interference studies. For investigating the effect of presence of heavy metal cations as impurities on the resolution of sugars from their mixture, 0.20 µl of the standard test mixture of sugar was spotted on the plate followed by the spotting of 0.20 µl of cations being considered as impurities. The plates were

developed with desired mobile phase, spots were detected with orcinol and hR_F values of the separated sugars were calculated.

4.2.12 *Optimization of mobile phase*

To examine the effect of other biosurfactants, sodium deoxycholate (NaDC) in mobile phase (M_7) was replaced by sodium cholate (M_{16}), sodium taurocholate (M_{17}) and chromatography of sugars was performed as done earlier.

To study the effect of nature of surfactants on the chromatographic behavior of sugars, sodium deoxycholate (NaDC) in mobile phase (M_7) was substituted by linear anionic surfactant, SDS (M_{18}), nonionic surfactant, Tw-20 (M_{19}) and cationic surfactant, CPC (M_{20}).

To further optimize the mobile phase M_7, acetonitrile was replaced with other organic solvents like methanol (M_{21}) and acetone (M_{22}).

4.2.13 *Limit of detection*

The limits of detection of all eight sugars were determined by spotting 0.02 µl of the concerned sugar solutions onto the chromatoplates, which were developed with M_7, NaDC:ACN (1:5 v/v) and the spots was visualized using detector. This was repeated with successive reduction of the concentration of sugar solution until no detection was possible. The procedure was repeated three times for each sugar. The amount of sugar just detectable in the M_7 was taken as limit of detection of that particular sugar.

4.2.14 *CMC by conductivity measurements*

ELICO conductivity bridge, model CM82T, and dip cell (cell constant 1.02 cm^{-1}) were employed to perform the conductivity measurements at 30 °C. Stock solution of sodium deoxycholate (24 mM) was prepared in ACN: DDW (5:1). The desired mole fractions of biosurfactant were obtained by mixing precalculated volume of the stock solution. The conductivity at each mole fraction of sodium deoxycholate

was measured by successive addition of stock solution of biosurfactant. A break in the conductivity vs. total concentration curve signals onset of micellization process.

4.3 RESULTS AND DISCUSSION

From the results of present study (**Tables 4.1, 4.2** and **Figures 4.2 - 4.4**) following trends are noticeable:

(i) With DDW (M_1) as eluent, all sugars co-migrate with solvent front (hR_F = 100) showing strong affinity towards the mobile phase as compared to the stationary phase. Thus, DDW is not useful as eluent for the separation of sugars.

(ii) In case of aqueous micellar bile salt solution (1% w/v, M_2), high mobility (hR_F = 98) showing broad and smeared spots for all sugars was observed and thus it is also not suitable for separation purposes.

(iii) With pure acetonitrile (M_3), all sugars remain at/near the point of application in the form of highly compact spots on the silica layer (hR_F = 00) showing strong interaction with the stationary phase and hence it is also not suitable for separating sugars.

Thus, mono-component eluents were found unsuitable for the separation of sugars and hence two-component mobile phase systems consisting of water plus acetonitrile (M_4) or aqueous sodium deoxycholate (1% w/v) plus acetonitrile in different volume ratios (M_5-M_{15}) were tested.

(iv) In water plus acetonitrile mixture (M_4, 1:5 v/v), all sugars were found to show little mobility without differential migration and hence no separation was possible.

On decreasing the concentration of acetonitrile from 90% (M_5) - 10% (M_{15}), none of the sugars shows a definite pattern regarding the variation of their mobility as a function of the change in volume fraction of acetonitrile (Figure 4.3). At or below 16.7 % v/v acetonitrile, mobility of all sugars remains unaltered. Addition of aqueous micellar sodium deoxycholate solution to acetonitrile leads to the increase

in the polarity of mobile phase which resulted in the differential mobility of sugars. Therefore, on the basis of above studies, TLC system comprising of aqueous (1%, w/v) sodium deoxycholate plus acetonitrile (M_7, 1:5, v/v) was identified as the most suitable for separation of disaccharides from hexose and pentose (**Figure 4.2**). It also appears that the mobility of sugars depends upon the number of hydroxyl groups in the molecule of sugars. The hR_F values were found to decrease with the increase in the number of hydroxyl groups in the molecule of sugars.

Order of hR_F values of three series of sugars (mentioned in parenthesis) was Pentoses (46 - 48) > Hexoses (30 - 33) > Disaccharides (09 - 18) which is in reverse order of the number of hydroxyl groups in the molecule of the sugars such as Order of magnitude of hydroxyl Groups: 4 (pentose) < 5 (hexose) < 8 (disaccharide).

Organic modifiers

The addition of organic modifier in aqueous surfactant has been found to influence the CMC value of the surfactant. In present study, we have used aqueous NaDC in combination with acetonitrile at concentration level of 24 mM. The CMC value calculated by us for the NaDC- acetonitrile mixture(M_7) was 2.49 mM. Thus, in present case NaDC at such higher concentration(about ten times higher than CMC value) is always present in micellar form irrespective of the proportion of acetonitrile in mixed mobile phase systems (**Figure 4.3**).

The substitution of acetonitrile with methanol (M_{21}) and acetone (M_{22}) in the mobile phase (M_7) does not improve the separation efficiency.

Effect of other surfactants

Substitution of sodium deoxycholate by other bile salts (NaTC, NaC) and ionic surfactants (SDS, CPC and Tw-20) in the mobile phase M_7 does not improve the chromatographic performance of mobile phase systems. In fact, all produce diffused spots with decreased resolution efficacy. The unique selectivity of NaDC towards sugars may be attributed to specific interactions compared to long-chain surfactant

aggregates.

In order to demonstrate the real effect of biosurfactant (i.e NaDC) on the separation efficacy of sugars, the separation of mixture of sugars was examined using selected mobile phase M_7 as well as the mobile phase water : acetonitrile (M_4, 1:5,v/v). From **Table 4.1**, it is evident that the mixture of sugars could not be resolved with M_6 (i.e. in the absence of NaDC) and thus the presence of NaDC in the mobile phase improves the chromatographic performance of the eluent.

The color intensity of detected spots on HPTLC plate remains unaltered for a month and hence the plates can be stored as record for the comparison in future.

Effect of metal cations as impurity

To examine the practicability of the mobile phase, M_7 and in view of the fact that metal cations are associated with biological systems, sugars were chromatographed with metal cations as foreign substances in the sample. Although cations such as Bi^{3+}, Cu^{2+}, Cr^{6+} and Ni^{2+} do not influence the separation but the cations like Co^{2+}, Hg^{2+}, Th^{4+}, Pb^{2+}, Tl^{+}, VO^{2+}, Zn^{2+} and Al^{3+} were found to hamper the separation (**Table 4.2**) due to their certain specific interactions with the sugars under study. To study the interference of heavy metals on separation of sugars is important from their physiological as well as environmental significance. Earlier studies [19-20] have also demonstrated the detrimental influence of transition as well as alkali/ alkaline earth metals on the chromatographic performance of sugars via the formation of coordination bonds between sugars and metal ion. Our observations are in consonance of the earlier studies.

Limit of detection

Sugars in the range can 40-50 ng/zone of all sugars could be easily detected with this system, below which the visualization of spots was not possible. Thus, the present procedure is very useful for the detection of nanogram amounts of sugars on HPTLC.

Standard deviation

The standard deviations in R_F values for sugars (C_1, C_3, C_4, C_7,C_8) and (C_2, C_5, C_6) were found to be 0.0075 and 0.0025 respectively indicating the reasonably good reproducibility of the proposed method.

4.4 CONCLUSION

In the present paper, the use of bile acids as additive in mobile phase for HPTLC is presented and evaluated. The proposal is practically evaluated by means of the separation of a mixture of disaccharides, hexose and pentose in pharmaceutical products and in human blood. The use of biological surfactants on mobile phase composition may allow reducing the volume of toxic organic solvent which is desirable from the environmental point of view.

4.5 APPLICATION

The practical utility of this method was examined by analysis of two commercially available pharmaceutical products (Becozinc and Honitus) and biological sample (blood serum). It was found that M_7 mobile phase was efficient and suitable for the identification of disaccharide in pharmaceutical formulations and biological systems after preliminary separation from pentose and hexose. It is evident from the chromatogram (**Figure 4.4**) showing three zones of separated sugars that the proposed TLC system is well suited for identification and separation of sugars.

REFERENCES

[1] A. Szabó, A. Kónya, I. Winkler, G. Máté, B. Erdélyi, *J. Planar Chromatogr.-Mod TLC* **19** (2006) 418.

[2] R. P. Pascale, A. A. Luc, *J. Planar Chromatogr.-Mod TLC* **4** (1991) 77.

[3] G. Mantovani, G.Vaccari, E.Dosi, G. Lodi, *Carbohydrate polymers* **37** (1998) 263.

[4] J.A. Jarusiewiez, B. Fried, J. Sherma, *J. Comp. Biochem. Physiol* B **145** (2006) 346.

[5] M. D. Maloney, Carbohydrates. In: Handbook of Thin Layer Chromatography (Vol. 89). Sherma J, Fried B (3rd Ed.) Marcel Dekker, Inc., New York, USA, 2003, 445.

[6] S.H. Nam, F.R. John, *Carbohydrate Research* **313** (1998) 135.

[7] R. Klaus, W. Fisher, E.H. Hauch, *Chromatographia* **29** (1990) 467

[8] R. W. Williams, Z. Fu, W.L. Hinze, *J. Chrom. Sci.* **28** (1990) 292.

[9] J. M. Herrero-Martinez, E. F. Simo-Alfonso, C. Mongay-Fernandez, *J. Chromatogr.* **895** (2000) 227.

[10] A.L. Crego, M.A. García, M.L Marina, *J. Microcolumn Separation* **12** (2000) 33.

[11] M. Skorka, M. Aszteinborska, *Colloids and Surfaces A. Physicochem. Eng. Aspects* **332** (2009) 1.

[12] A. Pyka, M. Dołowy, D.Gurak, *J. Liq. Chromatogr. Related Technol.* **28** (2005) 2273.

[13] R. O. Cole, M. J. Sepaniak, W. L. Hinze, J. Gorse, K Oldiges, *J. Chromatogr.* **557** (1991) 113.

[14] H. Ren, X. Li, M. Qi, C. Stathakis, N. J. Dovichi, *J. Chromatogr. A.* **817** (1998) 307.

[15] F. Momenbeik, J. H. Khorasani, *Acta Chromatographica* **16** (2006) 58.

[16] A. J. Alpert, *J. Chromatogr.* **499** (1990) 177.

[17] T. Ikegami, K. Tomomatsu, H. Takubo, K.Horie, N.Tanakka, *J. Chroamtogr A* **1184** (2008) 474.

[18] P.Hemström, K Irgum, *J. Sep. Sci.* **29** (2006) 1784.

[19] K.B. Kalinina, L.S. Litvinova, *Russ. J. Appl. Chem.* **74** (2001)1343.

[20] J. Flieger, H. Szumilo, *J. Planar Chromatogr.- Mod TLC* **14** (2001) 338.

Table 4.1: hR_F (R_F x 100) of sugars on HPTLC plates with different mobile phases

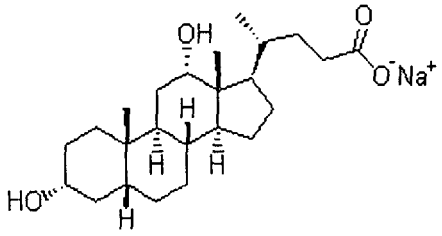
Code	Analyte							
	Pentose		Hexose				Disaccharide	
	C_5	C_6	C_1	C_2	C_3	C_4	C_7	C_8
M_1	100	100	99	98	96	96	94	98
M_2	98	96	95	95	94	97	96	94
M_3	00	00	00	00	00	00	00	00
M_4	33	32	25	28	24	30	13	11
M_5	38	40	18	22	06	14	04	19
M_6	50	49	32	33	17	30	18	38
M_7	46	45	28	29	10	24	05	34
M_8	54	56	44	45	29	42	29	47
M_9	50	52	40	41	25	38	25	42
M_{10}	67	70	70	65	53	63	53	69
M_{11}	57	57	55	55	44	55	44	54
M_{12}	95	95	95	95	95	95	95	90
M_{13}	79	79	70	69	75	74	65	75
M_{14}	98	98	98	95	95	95	98	98
M_{15}	98	98	98	95	95	98	95	98
M_{16}	45	41	25	28	11	25	13	24
M_{17}	60	57	55	48	56	55	66	66
M_{18}	48	46	33	27	30	32	18	17
M_{19}	75	75	62	63	58	61	50	66
M_{20}	DS	DS	39	40(T)	06	40(T)	06	40
M_{21}	71	75	69	67	72	70	66	71
M_{22}	68	61	63	56	23(T)	53	23(T)	58

DS – Diffused Spot T – Tailed Spot (R_L-R_T > 30)

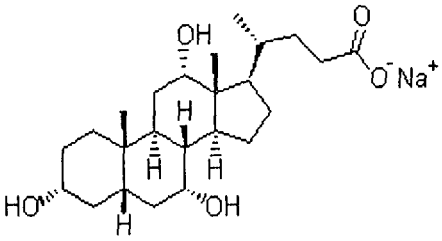
Table 4.2: Effect of metal cations on the separation of sugars

Sugars / Metal Cations	hR_F Values		
	Disaccharides	**Hexose**	**Pentose**
Al^{3+}	07	09	10
Bi^{3+}	07	26	34
$CrO4^{6+}$	07	24	33
Cu^{2+}	05	28	34
Co^{2+}	07	07	09
Hg^{2+}	10	90	90
Ni^{2+}	05	28	32
Pb^{2+}	09	09	08
Th^{4+}	10	07	07
Tl^{+}	00	10	06
VO^{2+}	00	10	06
Zn^{2+}	03	03	06

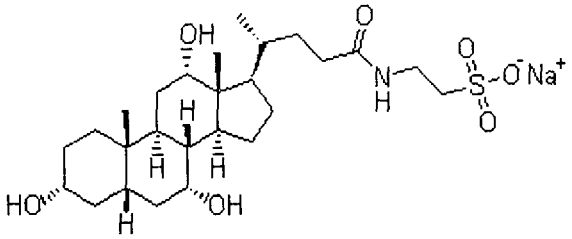
The hR_F values of disaccharide, hexose and pentose in the absence of metal ions were 18, 30 and 46 respectively



a) Sodium deoxycholate (3α, 12α-dihydroxy-5β-cholan-24-olic acid, sodium salt)



b) Sodium cholate (3α, 7α, 12α-trihydroxy-5β-cholan-24-olic acid, sodium salt)



c) Sodium taurocholate (2-[3α, 5β, 7α, 12α-trihydroxy-5β-cholan-24-yl] amino) ethanesulfonate)

Figure 4.1: Structures of bile salts a) sodium deoxycholate b) sodium cholate c) sodium taurocholate

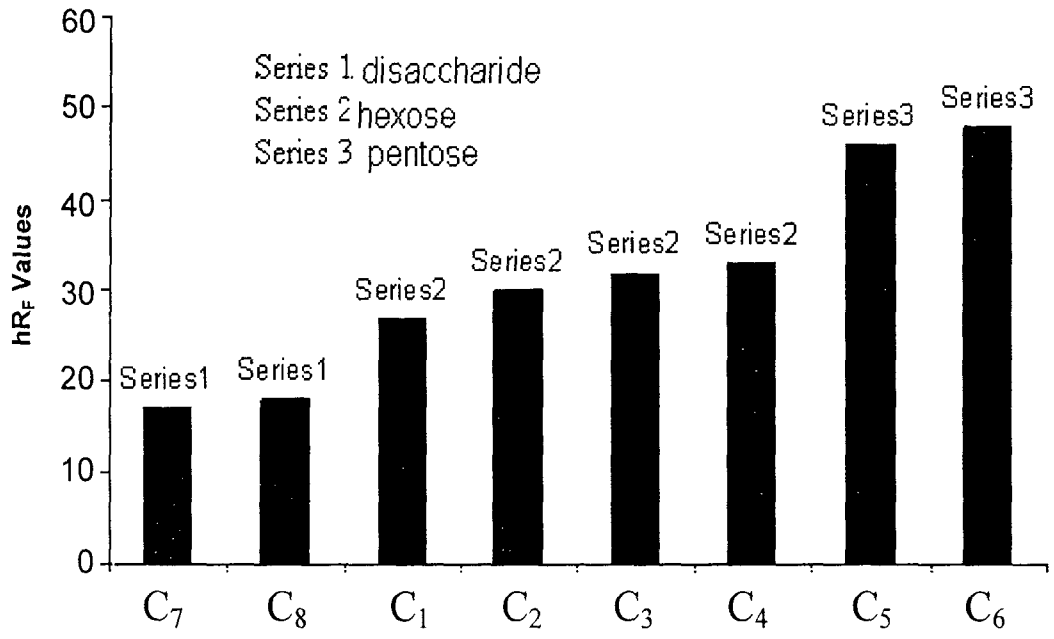


Figure 4.2: Plot Showing differential mobility of sugars using M_7 (NaDC-ACN, 1:5 v/v)

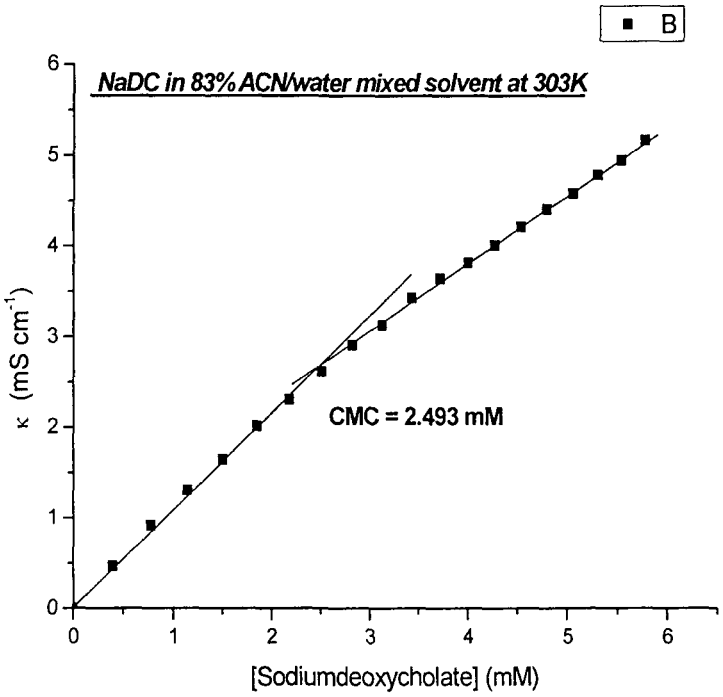


Figure 4.3: Plot of specific conductivity vs concentration of NaDC

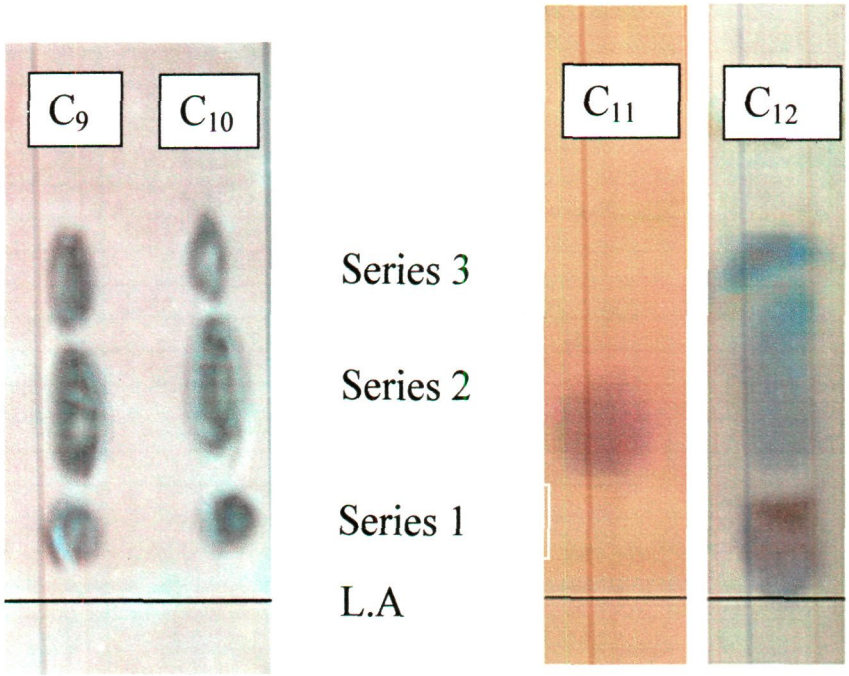


Figure 4.4: Chromatogram showing three zones of separated sugars in marketed formulations [Honitus (C_9), Becozinc (C_{10})] and human blood serum (C_{11} normal person, C_{12} diabetic patient) Ddveloped with M_7 (NaDC: ACN, 1:5 v/v)

L. A: Line of Application

Series 1: dissacharide

Series 2: hexose

Series 3: pentose

CHAPTER-5

Application of Water-in-Oil Microemulsion for Chromatographic Study of Different Groups of Organic Compounds

5.1 INTRODUCTION

The growing interest in Microemulsion (ME) or swollen-micelles as drug delivery vehicles arises mainly from their physicochemical properties such as transparency, low viscosity, and thermodynamic stability over a range of temperature interval and high solubilization capacity [1]. The possibility of increased solubility of sparingly soluble drugs in ME is of great interest because of the improved therapeutic efficacy of the drug, reduction in the volume of the vehicle and minimization of the *toxic side effects [2].*

The original application of oil-in-water ME in bioanalysis was reported by Berthod *et al.* when they separated a series of alkylbenzene and screened 11 drugs illegally used in sports [3]. A number of publications on the potential use of ME as a separation media in HPLC [4], micellar liquid chromatography [5], capillary electrophoresis [6] and microemulsion electrokinetic chromatography (MEKC) have appeared recently [7]. In addition, other application of ME is tertiary oil recovery, cosmetic, lubrication; enzyme catalysis, chemical synthesis etc. were also reported in literature [8, 9].

MEs contain nanometer-sized surfactant coated droplets of oil suspended in water, referred to as oil-in-water ME and vice-versa for water-in-oil ME. In ME liquid chromatography, the surfactant is added in the excess of the critical micelle concentration (CMC) which resulted in large amount of micelles in the mobile phase in which the analyte partitions within the micelles rather than adsorbs onto the stationary phase.

The versatile nature of ME generated renewed interest of chemists to utilize it in chromatographic procedures [10]. Literature suggests that MEs were used as mobile phase to investigate the retention behavior of nitroanilines, amino acids, herbal plant extracts, antibiotics etc. [11-14].

These micellar media seemed to offer another viable clean alternative to traditional organic solvents. Previous studies on thin layer chromatography (TLC) of sugar, amino acids, vitamins, lipids, dyes, purines and pyrimidines utilized hydro-organic,

mixed-organic and aqueous surfactants with organic additives as mobile phases for their separation and identification.

So, far no work has been reported for the identification of common sugars by HPTLC using water-in-oil ME as eluent. Furthermore, the present study also provides the general applicability of water-in-oil microemulsion in HPTLC analysis of compounds belonging to different groups such as vitamins, amino acids, purines and pyrimidines, lipids and dyes.

5.2 EXPERIMENTAL

All experiments were performed at 25 ± 2°C

5.2.1 *Apparatus*

High Performance Thin Layer Chromatography (HPTLC) plates and TLC twin-trough chamber Camag, Muttenz, Switzerland lined with lid were used. A glass sprayer was used to spray reagent on the plates to locate the position of the spot of analyte.

5.2.2 *Reagents*

n-Pentanol (Acros organics, New Jersey, USA), heptane and CTAB were of CDH, India. Amino acids (D- glutamic acid, L–serine, D–alanine, L–tyrosine, L–histidine, L–lysine, L–arginine and phenylalanine), sugars (dextrose, D-fructose, D-lactose, D-galactose, D-ribose and D-xylose), lipids (lauric acid, palmitic acid, stearic acid, n-butyric acid and oelic acid), vitamins (thiamine, riboflavin and pyridoxine) and dyes (xylenol orange, bromocresol green, alizarin red S, congo red, pyrocatechol violet, malachite green, methylene blue, brilliant green, rhodamine B and crystal violet) were from CDH, India. Purines (alanine and guanine) and pyrimidines (cytosine, thymine and uracil) were obtained from Himedia (Mumbai), India. All chemicals were of analytical grade.

5.2.3 *Preparation of test solutions*

Aqueous (1% w/v) of amino acids, sugars and B-complex vitamins were prepared. In case of pyridoxine and riboflavin few drops of NaOH were added to make clear solution. Purines and pyrimidines (1% w/v) were prepared in water-methanol mixture (60:40, v/v) and few drops of ammonia were added when necessary. Lipids (1% w/v or v/v) in chloroform: ethanol (1:1) was prepared. Dyes (0.1%) were prepared in mixture of double distilled water plus ethanol in 1:1 ratio by volume.

5.2.4 *Marketed formulations*

Becasules* capsules (B-complex Forte with vitamin C), Pfizer and Becozinc syrup, Dr. Reddy's, Hyderabad, India, analysed by the proposed method.

5.2.5 *Preparation of formulation solutions*

Becasules* capsule was finely powdered and dissolved in 10 ml of double distilled water (B_1 sample) and 1 % solution of Becozinc syrup was prepared in double distilled water(B_2 sample).

5.2.6 *Detectors*

Ethanolic solution (0.3%) of ninhydrin was sprayed on the HPTLC plates to locate the position of amino acids. Purple spots appeared on heating the HPTLC plates at 60°C for few min.

Sugars were detected by spraying ethanolic orcinol solution on silica HPTLC plates and heating at 110°C for 5-10 min. The detected spots appeared as brown- purple for all disaccharides and hexoses except D-fructose (appeared as orange) and blue for pentoses.

All vitamins, purines, pyrimidines and lipids were UV active and can be easily visualized under short UV lamp. Dyes were detected visually according to their original color.

5.2.7 *Stationary phase*

Silica gel 60 F_{254} HPTLC (1.05715.001) and PEI Cellulose F_{254} (1.05579.0001) plates were used as stationary phases.

5.2.8 Mobile phase

Water-in-oil ME prepared at 25°C by titrating a coarse emulsion of heptane (160 ml), water (8 ml) and CTAB (8 gm) with n-pentanol (25 ml). The ME system was transparent optically clear and remained stable at 25 ± 2°C for several weeks. This was used as mobile phase for six groups of organic compounds.

5.2.9 *Procedure*

Test solutions (1µl) of all analytes were applied on (6 cm x 6 cm) silica gel 60 F_{254} and PEI cellulose high-performance thin layer plates with the help of micropipette at about 1 cm above the lower edge of the plates. The solvent ascent was fixed to 5 cm in all cases for the determination of R_F value of individual analyte. Linear ascending development was carried out in vapor equilibrated Camag TLC twin-trough chamber. The optimized chamber saturation time for the mobile phase was 10 min. at room temperature (25 ± 2°C). Followed by the development, TLC plates were dried at room temperature. The plates were detected by using appropriate detector for desired analyte. The R_L (R_F of leading front) and R_T (R_F of trailing front) values of each spot were determined and the R_F value was calculated.

For the separation of vitamins or dyes from their mixtures, equal volumes of vitamins or dyes were mixed and 1 µl of the resultant mixture was applied on TLC silica gel or cellulose plate. The plate was developed with mobile phase, the spots were detected and the R_F values of the separated spots of vitamins and dyes were calculated. Similarly, dyes were also separated on cellulose plates.

For investigating the interference of other bioorganic molecules (amino acid, sugars, lipids, purines and pyrimidines) on the mobility and mutual separation of

vitamins, aqueous solutions (as prepared earlier) were used for interference studies. The effect of biomolecules as impurities on the resolution of vitamins from their mixture, 1 µl of the standard test mixture of vitamins was spotted on the plate followed by the spotting of 1 µl of biomolecules considered as impurities. The plates were developed with microemulsion containing CTAB mobile phase, spots were detected under UV lamp and R_F values of the separated vitamins were calculated.

5.3 RESULTS AND DISCUSSION

Chromatography of six groups of organic compounds (amino acids, vitamins, sugars, lipids, dyes, purines and pyrimidines) was performed with water-in-oil microemulsion containing CTAB as eluent on two different stationary phases i.e. silica gel G and cellulose HPTLC plates. The following trends were noticed.

(i) All amino acids were strongly retained on both silica and cellulose plates and thus resolution of amino acids with microemulsion as mobile phase is not possible.

(ii) All polar sugars, co-migrate without any possible separation on silica gel HPTLC plates showing low mobility ($R_F = 0.29$).

(iii) Alike amino acids, polar purines and pyrimidines show high affinity towards both the adsorbents (silica gel and cellulose) and cannot be resolved from their mixtures.

(iv) All lipids being hydrophobic well moved with elution window on silica gel G or cellulose showing significant mobility ($R_F = 0.98$) and thus imposing the restriction on their mutual separation. However, they can be easily separated from other organic compounds (amino acids, vitamins, sugars, dyes, purines and pyrimidines) having lower R_F value.

(v) A fair separation of B-complex vitamins (thiamine hydrochloride, riboflavin and pyridoxine hydrochloride), is always possible on silica HPTLC plate with water-in-oil ME system. For these vitamins, the order of R_F values, given in parenthesis, was thiamine (0.14) < pyridoxine (0.27) < riboflavin (0.44).

The effect of impurity of other bioorganic molecules (lipids, amino acids, sugars and purines & pyrimidines) on the separation of vitamins was also examined. In the presence of lipids, sugars purines and pyrimidines, the mutual separation is always possible but uracil is the exception which hampers the separation. Amongst amino acids, glutamic acid and tyrosine hurt the mutual separation by producing a singly tailed spot near the point of application as a result of co- migration of these vitamins (thiamine, pyridoxine, riboflavin) in the sample. However, in the presence of other amino acids, all the three vitamins are well resolved on single TLC plate. Thus, for vitamins silica gel (inorganic adsorbent) is more efficient stationary phase compared to cellulose (organic adsorbent) because on PEI cellulose HPTLC layers, the separation of these three vitamins could not be achieved. The developed method is applicable to identification of B-complex vitamins in marketed formulation as evident from results presented in **Table 5.1**.

(vi) All dyes were found to stay near the point of application on silica HPTLC plates and hence they cannot be separated. However, a selective separation of bromocresol green (R_F = 0.89) is possible from all cationic (malachite green, methylene blue, brilliant green, rhodamine B and crystal violet) and anionic (xylenol orange, bromocresol green, alizarin red S, congo red, pyrocatechol violet) dyes ($R_F \approx 0.01$) on cellulose HPTLC plate.

Almost all the analytes produce well compact spots near or at the point of application on silica HPTLC, which shows a strong affinity of polar analytes with polar silica gel. Low mobility of polar analytes may be attributed to their strong attraction to negative silanol groups on the silica gel surface [15].

5.4 CONCLUSION

Oil-in-water microemulsion proved to be useful surfactant-mediated system for ternary separation of vitamins (thiamine, riboflavin and pyridoxine) in pharmaceutical preparations. On cellulose layers, bromocresol green was selectively separated from other anionic as well as cationic dyes.

REFERENCES

[1] P. K. Ghosh, R. S. R. Murthy, *Drug. Del. C.* **3** (2006) 167.

[2] R. Ryan, S. Donegan, J. Power, E. McEvoy, K. Altria, *Electrophoresis* **30** (2009) 65.

[3] A. Berthod, J. J. Lasesna, I. Carretero, *J. Liq. Chromatogr. Related Technol.* **15** (1992) 3115.

[4] K. Altria, M. Broderick, S. Donegan, J. Power, *Chromatographia* **62** (2005) 341.

[5] M. B. Simon and D. A. Kevin, *J. Sep. Sci.* **27** (2004) 1498.

[6] A. Marsh, M. Broderick, K. Altria, J. Power, S. Donegan, B. Clark, *Methods Mol. Biol.* **384** (2008) 205.

[7] M. Murillo–Arbizu, E. Gonzatez–Penas, S. H. Hansen, S. Amezqueta, J. Ostergaard, *Food Chem. Toxicol.* **46** (2008) 2251.

[8] B. K. Paul, S. P. Moulik, *Curr. Sci.* **80** (2001) 990.

[9] K. D. Altria, *J. Chromatogr. A* **892** (2000) 171.

[10] G. M. Eccleston, Emulsion and Microemulsions, (IInd Ed., J. Swarbrick, J.C. Boylan, Marcel Dekker, Inc., New York, (2002) 1080.

[11] A. Mohammad, S. Hina, *Acta Chromatographica* **15** (2005) 238.

[12] A. Mohammad, V. Agrawal, S. Kumar, *J. Planar Chromatogr.* **16** (2003) 220.

[13] S. F. Cui, B. Q. Fu, F. S. C. Lee, X. R. Wang, *J. Chromatogr. B* **828** (2005) 33.

[14] A. Malenovic, D. Ivanovic, M. Medenica, B. Jancic, S. Markovic, *J Sep. Sci.* **13** (2004) 1087.

[15] B. A. Olsen, *J. Chromatogr. A* **913** (2001) 113.

Table 5.1: Identification of Vitamins on Silica HPTLC plates using Water-in-Oil ME containing CTAB

	R_F Value		
Sample	Thiamine	Riboflavin	Pyridoxine
B_1	0.08	0.27	0.40
B_2	0.08	0.27	0.41

CHAPTER-6

Sodium Deoxycholate Micelles Activated Separation of Coexisting Five-Nucleobases by High-Performance Thin Layer Chromatography

6.1 INTRODUCTION

High Performance Thin Layer Chromatography (HPTLC) is well suited to the separation of nucleobases. Most of reports on this topic are limited to the use of PEI-cellulose, ODS (octadecyl silica), and silica gel as layer materials [1-3]. Chiral plates have also been used for separation of nucleobases, and enantiomers [4]. The first use of micellar mobile phase by Armstrong in 1979 for the analysis of nucleotides was 1.3 AOT in cyclohexane-water mixture [5]. The suggested mobile phase was capable to resolve a mixture of nucleotides on silanized silica gel. Compared to normal micelle forming surfactants, bile salts are unique in forming helical aggregates in solution [6-8]. In nature, sodium deoxycholate **(Figure 6.1)** referred as "secondary bile acid" is produced in the intestine from the salts of glycocholic, and taurocholic acid by the action of bacterial enzymes. Applications of sodium deoxycholate range from cell lyses, liposome preparation, isolation of membrane proteins and lipids, a cell culture media as supplement, preventing non specific binding in affinity chromatography, micellar electrokinetic chromatography and other chromatographic techniques [8-11]. Versatility of bile salts led us to utilize its analytical potential as mobile phase for the separation of coexisting nucleobases from their mixtures. This is probably the first report to separate five nucleobases (two purines, and three pyrimidines) on cellulose 60 F_{254} HPTLC plates by utilizing sodium deoxycholate (aqueous 5.0 %) plus acetonitrile (1:1, v/v) as mobile phase. Furthermore, we have successfully achieved an interesting separation of thymine from uracil. This separation is important because both pyrimidines are used to differentiate between the structures of DNA and RNA (**Figure 6.2**).

6.2 EXPERIMENTAL

All experiments were performed at 25 ± 2°C

6.2.1 *Apparatus*

Pre-coated cellulose 60 F_{254} HPTLC aluminum foils were of Merck, Darmstadt, Germany. TLC twin-trough chamber Camag, Muttenz, Switzerland lined with lid were used.

6.2.2 *Reagents*

Acetonitrile (Lichsolv, Merck, India), purines and pyrimidines (Himedia, Mumbai, India) and sodium deoxycholate (E-Merck, India) were used.

All chemicals and reagents were of analytical reagent (AR) grade. Double distilled water (DDW) was used throughout the experiment.

6.2.3 *Test solutions*

Solutions of purines (adenine, and guanine), and pyrimidines (cytosine, thymine, and uracil) were prepared in a mixture of methanol and water (4: 6) to give concentration of 1.0% w/v.

6.2.4 *Detector*

HPTLC plates containing fluorescent indicator were kept under short wave 254nm UV light to locate the position of analyte.

6.2.5 *Stationary phases*

Cellulose 60 F_{254}, silica gel 60 F_{254}, RP-18 F_{254} and Kieselgel 60 F_{254} aluminium foils of size 6 cm x 6 cm were used as stationary phases.

6.2.6 *Buffer solutions*

The buffer solutions used to prepare the solution of NaDC (5.0%) at different pH levels are listed in **Table 6.1**.

6.2.7 *Mobile phases*

A variety of mobile phases listed in **Table 6.2** were examined to identify the most suitable solvent system for separation of nucleobases.

6.2.8 *Procedure*

Precoated HPTLC plates were activated at 60 ± 2°C in an electrically controlled oven for 20 min and stored in closed chamber until used. Spotting of 1.0 µl of sample using micropipette (Tripette 0783178, Germany) is done at 1.0 cm from the base of HPTLC plates. Spots were air dried and developed in a closed presaturated Camag TLC twin-trough chamber with desired mobile phase by ascending technique up to the ascent of 5.0 cm from the point of application. After development, the HPTLC plates were withdrawn from glass chamber, air dried and then detected under UV light to locate the position of analyte as fluorescent spots under short wavelength.

The R_F values of visualized spots were calculated by using formula:

$$R_F = 0.5\ (R_L + R_T)$$

where $R_L = R_F$ of the leading edge, and $R_T = R_F$ of trailing edge.

In order to examine the effect of volume of acetonitrile added in aqueous NaDC, R_F of nucleobases, were chromatographed on cellulose layers. The affinity of the solvent systems with different concentrations of aqueous NaDC was assessed by calculating the capacity factor values.

The capacity factor (K) was determined as a function of the conventional mobility R_F

$$K = \left(\frac{1}{R_F} - 1 \right)$$

It is used to indicate the relative affinity of purines or pyrimidines between the solid substrate and the solvent. Strong affinity for the substrate is indicated by high value of capacity factor and vice versa.

To study the effect of interference of mono- and bivalent cations or anions, urea, and EDTA on the R_F value of nucleobases, analyte sample (1.0 µl) was spotted onto

the activated TLC plates followed by the spotting of 1.0 µl of the interfering species on the same spot. The plates were developed with M_{10} (5% aqueous NaDC-AcN, 1:3, v/v). After development, spots were visualized under short wave 254nm UV light. The R_F valued were determined and compared with those obtained in the absence of interfering species.

For the separation, equal volumes of all five nucleobases were mixed and 1.0 µl of the resultant mixture was applied on HPTLC plates. The plate was developed with M_{10}, the spots were visualized and R_F values of the separated spots of the nucleobases were calculated.

The limits of detection of nucleobases were determined by spotting a definite volume of different concentrations of nucleobases (0.01-1.0 %) on the cellulose F_{254} HPTLC plates. The plates were then developed and the corresponding spots were detected. The method was repeated with successive lowering of the amounts of nucleobase (purine or pyrimidine) until no spot was detected. The minimum amount of nucleobase that could be visualized was taken as the limit of detection.

6.3 RESULTS AND DISCUSSION

Selection of mobile phase

The aim of the present study was to select a useful solvent system to achieve the separation of coexisting five nucleobases (two purines, and three pyrimidines). For this, purpose chromatography of all nucleobases was performed on cellulose layers using different solvent systems. Both mono- component as well as binary mixed solvent systems was tested for the chromatography of these five nucleobases. When micellar solutions of cationic (cetyltrimethylammonium bromide, CTAB), non-ionic (Tween-20), and anionic (sodium dodecyl sulfate, SDS) surfactants were used as eluent, all nucleobases show smeared spots with high mobility. Mixed micellar solutions were also not successful for separating nucleobases; all show broadened spots. In view of these unfavorable results, linear anionic surfactant, SDS was

replaced with helical sodium deoxycholate (bile salt). Aqueous 5.0% solution of sodium deoxycholate (NaDC) was selected for study because of its versatile nature of separating many isomeric compounds. It is well known that, the separation efficiency of pure micellar mobile phase system is improved by the addition of the organic additives [12]. Therefore, mixed mobile phases of 5.0% NaDC-acetonitrile in different ratio was tested, and the obtained results with these solvent systems are listed in **Table 6.3**. Addition of acetonitrile increases the hydrophobic nature of the mobile phase in order to retain the hydrophilic species. From the data of this Table, the following conclusions may be drawn:

- In mobile phase, M_1 (DDW) both purines show lower mobility compared to pyrimidines on cellulose 60 F_{254} layers.
- When 5.0%aqueous bile salt, NaDC (M_2) was used as the mobile phase, the R_F of all nucleobases slightly reduced as compared to their mobility in M_1.
- In both M_1, and M_2, the R_F of nucleobases was in the order: adenine < guanine < cytosine < thymine ≈ uracil.
- The R_F value of adenine, guanine, cytosine, thymine, and uracil was found to increase with the increase in volume ratio of acetonitrile in its mixture with 5.0%aqueous NaDC (M_3-M_6), followed by decline in R_F value from to M_{12}.

As more compact spots, and differential R_F were realized in M_{10} (5.0% aqueous NaDC-acetonitrile, 1:3). This mobile phase was selected for further studies. To understand the effect of other organic additives on the R_F values of nucleobases, acetonitrile in M_{10} was replaced by other organic solvents (viz: acetone, methanol, formic acid, and DMSO), and the resultant mobile phase systems (M_{13}-M_{16}) were used for chromatography.

- In M_{13}, and M_{14}, all nucleobases showed lower mobility as compared to M_{10}. The order of R_F was: $M_{10} > M_{13} > M_{14}$.
- In M15, and M16 all nucleobases showed constant and high R_F value (R_F > 0.90). Compact spots were realized in M_{15}, whereas in M_{16} spots were diffused. For investigating the effect of other bile salts, NaDC in M_{10} was

replaced by other biological bile salts (i.e. NaC or NaTC), and the resultant mobile phase systems (M_{17} and M_{18}) were used for chromatography. It was found that there was no significant change in R_F values on substitution of NaDC by NaC or NaTC in M_{10}.

The R_F data of nucleobases obtained with buffered 5.0% NaDC (pH 2.3, 3.4, 5.7, 7.2 or 11.9) plus acetonitrile (M_{19}-M_{23}) mobile phases are compared and presented in Figure 3. It is clear from this Figure that the R_F value of nucleobases is slightly influenced by pH of NaDC in all mobile phases (M_{19}-M_{23}), except guanine, which was not detected in M_{22}, and M_{23}. The R_F value of guanine was increased to 0.83 from its standard R_F value (0.12) in acidic media (M_{19}-M_{21}).

Effect of nature of sorbent layers

To establish the effectiveness of cellulose 60 F_{254} HPTLC layers, the retention behavior of nucleobases was also examined on different sorbent layers, and the obtained results have been plotted in Figure 4. Although more compact spots were realized on RP-18 F_{254} layers, but separation of coexisting five nucleobases is possible only on cellulose 60 F_{254} layers.

Effect of interferences

Table 6.4 summarizes the effect of a variety of impurities on retention (R_F) of nucleobases. From the data listed in **Table 6.4**, it is evident that the R_F values of nucleobases were altered from its standard value in the presence of impurities in the sample. All nucleobases showed high R_F value in the presence of Li^+ as compared to other alkali or alkaline earth metal cations. Guanine, cytosine, thymine and uracil were not detected in the presence of Mg^{2+}. The R_F value (retention) of nucleobases was greatly influenced in the presence of transition metal cations, probably due to greater affinity of transition metal cations to bind with DNA or RNA nucleobases. In the presence of Ni^{2+} and Cu^{2+}, all nucleobases showed almost similar R_F values. Zn^{2+} converted the compact spot of nucleobases into a badly diffused spots. The detection of guanine cytosine, thymine, and uracil in the presence of Co^{2+} was

difficult.

However, a significant increase in R_F values of adenine, guanine, and cytosine was noted in the presence of anions, urea, and EDTA. Both purines (adenine, and guanine) produced double spots in the presence of MoO_4^{2-} showing effective complexing tendency of molybdate ion.

Compared to adenine, the R_F value of guanine was greatly modified in the presence of impurities (cations, anions, urea,or EDTA) in the sample. This might be due to the presence of two attractive centres in guanine to interact with impurities, whereas in the case of adenine there is only one attractive centre [13].

Separation

The position of spots appeared on cellulose fluorescent plate is depicted in **Figure 6.5**. It is clear that, with mobile phase M_{10} (5% aqueous NaDC + AcN, 1: 3, v/v) purines and pyrimidines could be separated easily from their mixtures.

Limit of detection

The lowest possible detectable microgram amounts of all five nucleobases obtained on cellulose F_{254} HPTLC plates developed with M_{10} was ≈ 0.054 μg $spot^{-1}$. It shows that the developed method is reasonably suitable for identifying these nucleobases at trace level.

6.4 CONCLUSION

The proposed thin layer chromatographic system comprising of cellulose 60 F_{254} as stationary phase and 5.0% aqueous sodium deoxycholate (NaDC)-acetonitrile (AcN), 1:3 (v/v) phase is as mobile most favorable for the separation of coexisting five-nucleobases (adenine, guanine, cytosine, thymine, and uracil). It is highly selective, reliable, and rapid requiring 5.0–7.0 min. for resolution of DNA and RNA nucleobases.

REFERENCES

[1] K. Randerath, H. Struck, *J. Chromatogr.* **6** (1961) 365.

[2] K. E. Bij, M. Lederer, *J. Chromatogr.* **268** (1983) 311.

[3] J. J. Steinberg, O. G. Cajigas, *Chromatogr. Sci. Ser.*, New York, Wiley Press **89** (1996) 733.

[4] H. Hatzack, S. K. Rasmussen, *J. Chromatogr. B* **736** (1999) 221.

[5] D. Armstrong, R.Q .Terril, *Anal. Chem.* **51** (1979) 2160.

[6] E. Giglio, S. Loreti, N.V. Pavael, *J. Phys. Chem.* **92** (1988) 2858.

[7] A. R. Campanelli, S. C. De Sanctis, E. Giglio, N.V. Pavel, C. Quagliata, *J. Inclusion Phenomena* **6** (1989) 391.

[8] R. W. Williams, Z. Fu, W. L. Hinze, *J. Chromatogr. Sci.* **28** (1990) 292.

[9] A. F. Hofmann, K. J. Mysels, *Collo. Surf.* **30** (1988) 145.

[10] C. O. Thompson, V.C. Trenerry, B. Kemmery, *J. Chromatogr A.* **694** (1995) 507.

[11] W. A. Morgan, N. K. Thanh, Y. Ding, *J. Pharmaco.l Toxicol. Methods.* **57** (2008) 70.

[12] E. G. Sumina, S. N. Shtykov, N. V. Tyurina, *J. Anal. Chem.* **58** (2003) 720.

[13] J. V. Barda, J. Sponer, P. Hobza, *J. Phys. Chem.* **100** (1996) 7250.

Table 6.1: Buffer solutions used as solvents to prepare mobile phases containing 5.0 % NaDC

Composition	Volume ratio	pH
0.04M boric acid + 0.04 M phosphoric acid	50 : 50	2.3
0.04M boric acid + 0.04 M phosphoric acid + 0.24 M NaOH	50 : 50 : 8	3.4
0.04M boric acid + 0.04 M phosphoric acid + 0.24 M NaOH	50 : 50 : 10	5.7
0.04M boric acid + 0.04 M phosphoric acid + 0.24 M NaOH	50 : 50 : 14	7.2
0.04M boric acid + 0.04 M phosphoric acid + 0.24 M NaOH	50 : 50 : 60	11.9

Table 6.2: Solvent systems used as mobile phases in chromatographic studies

Code for mobile phases	Composition
M_1	DDW (double distilled water)
Aqueous micellar bile salt solution	
M_2	5.0% NaDC in DDW
Aqueous micellar solutions with organic additives	
M_3	M_2–AcN (9 : 1, v/v)
M_4	M_2–AcN (8 : 2, v/v)
M_5	M_2–AcN (7 : 3, v/v)
M_6	M_2–AcN (6 : 4, v/v)
M_7	M_2–AcN (5 : 5, v/v)
M_8	M_2–AcN (4 : 6, v/v)
M_9	M_2–AcN (3 : 7, v/v)
M_{10}	M_2–AcN (2.5 : 7.5, v/v)
M_{11}	M_2–AcN (2 : 8, v/v)
M_{12}	M_2–AcN (9 : 1, v/v)
M_{13}	M_2–Acetone (2.5 : 7.5, v/v)
M_{14}	M_2–MeOH (2.5 : 7.5, v/v)
M_{15}	M_2–HCOOH (2.5 : 7.5, v/v)
M_{16}	M_2–DMSO (2.5 : 7.5, v/v)
M_{17}	5.0% aqueous NaC–AcN (2.5 : 7.5, v/v)
M_{18}	5.0% aqueous NaTC–AcN (2.5 : 7.5, v/v)
Buffer micellar NaDC solutions with AcN (2.5 : 7.5, v/v)	
M_{19}	5.0% NaDC (pH 2.3)–AcN
M_{20}	5.0% NaDC (pH 3.4)–AcN
M_{21}	5.0% NaDC (pH 5.7)–AcN
M_{22}	5.0% NaDC (pH 7.2)–AcN
M_{23}	5.0% NaDC (pH 11.9)–AcN

AcN, acetonitrile; NaC, sodium cholate; NaTC, sodium taurocholate; MeOH, methanol; DMSO, dimethylsulfoxide.

Table 6.3: R_F values of five nucleobases on cellulose 60 F_{254} HPTLC plates developed with mobile phases (M_1-M_{18})

Mobile phase	Nucleobases				
	Purines			Pyrimidines	
	Adenine	Guanine	Cytosine	Thymine	Uracil
M_1	0.33T	0.57	0.78	0.80	0.80
M_2	0.50	0.54	0.60	0.70	0.70
M_3	0.53	0.50	0.75	0.75	0.74
M_4	0.55	0.48	0.75	0.80	0.80
M_5	0.56	0.50	0.75	0.90	0.86
M_6	0.60	0.57	0.80	0.89	0.86
M_7	0.58	0.48	0.77	0.85	0.82
M_8	0.55	0.40	0.71	0.85	0.77
M_9	0.49	0.24	0.58	0.85	0.75
M_{10}	0.44	0.12	0.50	0.84	0.72
M_{11}	0.44	0.10	0.48	0.84	0.73
M_{12}	0.12	0.00	0.14	0.53	0.41
M_{13}	0.32	0.10	0.46	0.67	0.38T
M_{14}	0.13	0.06	0.35	0.38	0.20
M_{15}	0.95	0.94	0.95	0.97	0.96
M_{16}	0.90	0.90	0.91	0.92	0.91
M_{17}	0.64	0.24	0.75	0.93	0.91
M_{18}	0.56T	0.18	0.70	0.83	0.80T

Each value is an average of four measurements; T = Tailed spot; N.D. = Not Detected.

Table 6.4: R_F values of nucleobases on cellulose 60 F_{254} HPTLC plates developed with mobile phase, M_{10} in the presence of organic and inorganic impurities in the working standard sample

Interfering ions	Nucleobases				
	Purines		Pyrimidines		
	Adenine	Guanine	Cytosine	Thymine	Uracil
Without impurities	0.44	0.12	0.50	0.80	0.80
Cations (mono- and bivalent)					
Li^+	0.60	0.82	0.74	0.70	0.70
Na^+	0.39	0.32	0.50	0.75	0.74
Mg^{2+}	0.36	N.D	N.D	0.80	0.80
Ba^{2+}	0.55	0.43	0.66	0.90	0.86
Co^{2+}	0.00	N.D	0.81	0.89	0.86
Ni^{2+}	0.77	0.77	0.70	0.85	0.77
Cu^{2+}	0.83	0.79	0.85	0.85	0.82
Zn^{2+}	0.54	0.47	0.60	0.85	0.75
Cd^{2+}	0.58	0.45	0.69	0.84	0.72
Pb^{2+}	0.59	N.D	0.00	0.84	0.73
Anions (mono- and bivalent)					
Br^-	0.56	0.38	0.14	0.53	0.41
CH_3COO^-	0.54	0.39	0.46	0.67	0.38T
NO_3^-	0.50	0.39	0.35	0.38	0.20
CO_3^{2-}	0.49	0.40	0.95	0.97	0.96
IO_4^-	0.54	0.41	0.91	0.92	0.91
SO_4^{2-}	0.50	0.41	0.75	0.83	0.86
MoO_4^{2-}	0.45	0.43	0.73	0.80	0.76
Complexing ligands					
Urea	0.55	0.40	0.70	0.82	0.77
EDTA	0.51	0.38	0.66	0.81	0.74

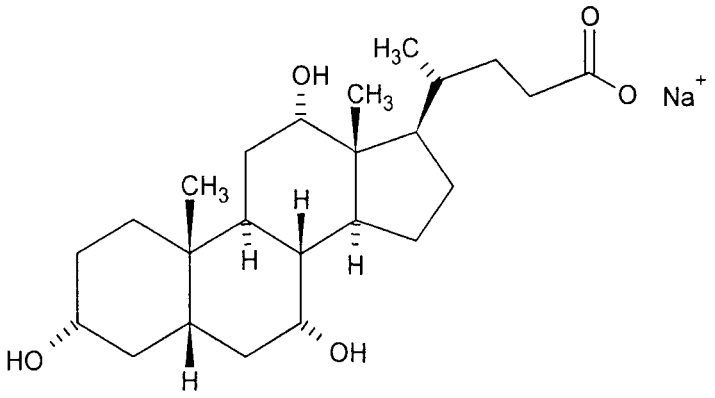
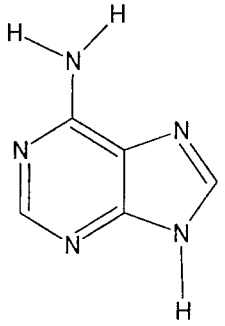
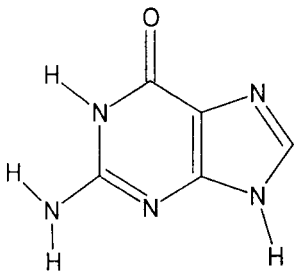


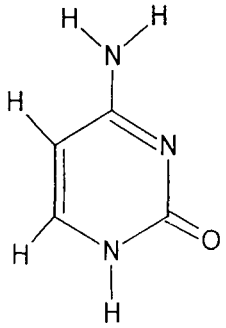
Figure 6.1: Structure of sodium deoxyycholate



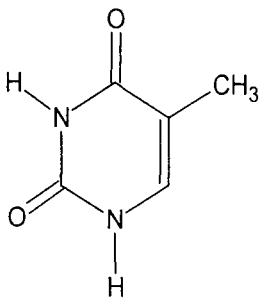
Adenine (pKa = 9.8)



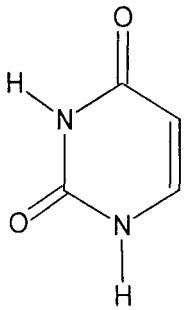
Guanine (pKa = 9.2, 12.3)



Cytosine (pKa = 12.2)



Thymine (pKa = 9.9)



Uracil (pKa = 9.5)

Figure 6.2: pKa value and chemical structure of nucleobases.

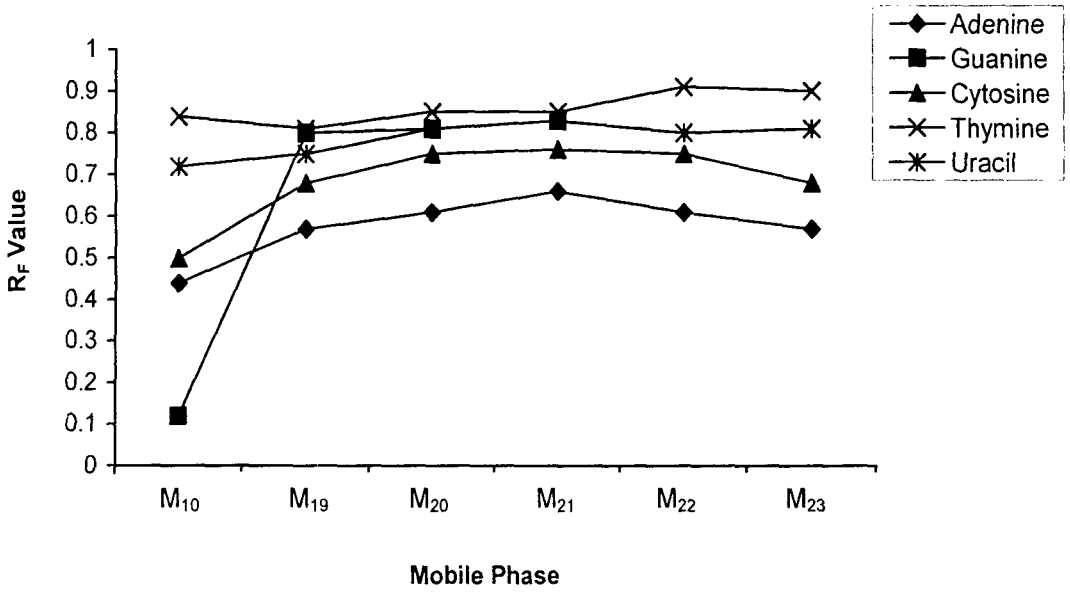


Figure 6.3: R_F value of five nucleobases on cellulose layers developed with micellar buffer mobile phases (M_{19}-M_{23})

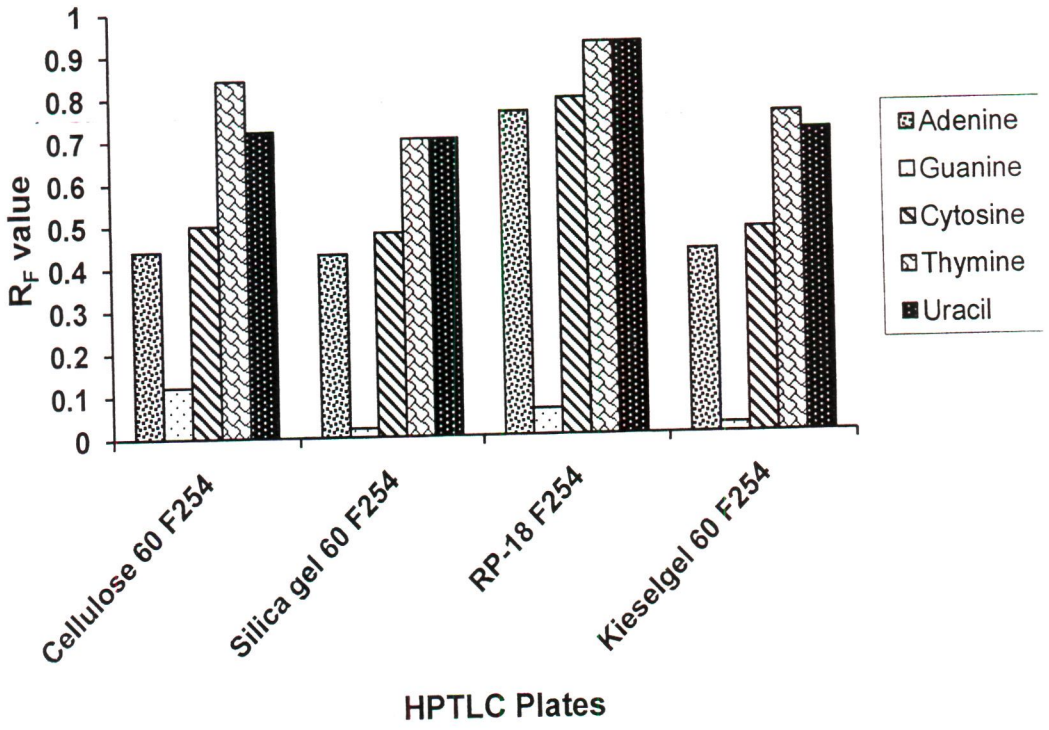


Figure 6.4: R_F values of nucleobases on various HPTLC plates developed with mobile phase, M_{10}

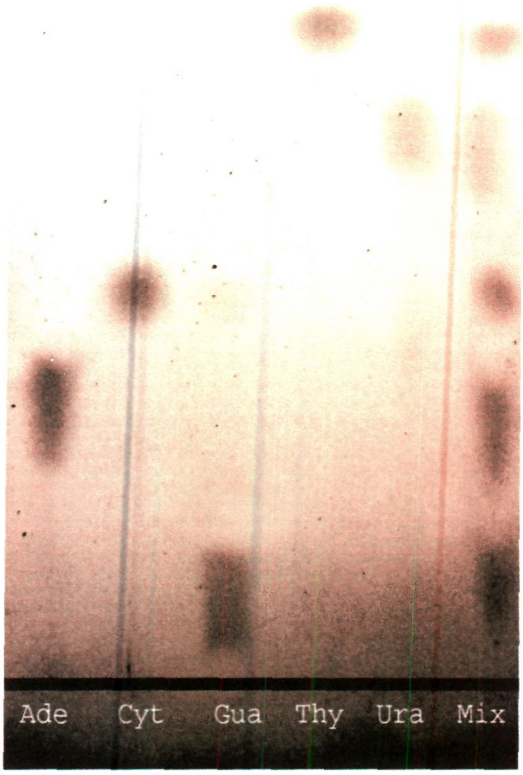


Figure 6.5: Separation of five coexisting nucleobases (adenine, cytosine, guanine, thymine, and uracil on cellulose 60 F_{254} layer developed with mobile phase M_{10}

Journal of Planar Chromatography

2007

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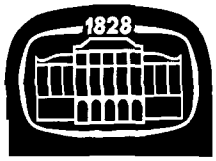
Vol. 20

SEPARATUM



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Editorial correspondence

A. Kakuk, Research Institute for Medicinal Plants

P.O. Box 11, H-2011 Budakalász, Hungary,

Phone: +36 26 340 533/204, Fax: +36 26 343 195, E-mail: jpc@akkrt.hu

Mixed Surfactants Enable Separation of Lysine from Other Essential Amino Acids in TLC on Silica Gel

Ali Mohammad* and **Sameen Laeeq**

Key Words

Thin-layer chromatography
Separation
Essential amino acids
Lysine
Mixed surfactants

Summary

Thin-layer chromatography of eight essential amino acids has been performed on silica layers with seventeen mobile phases of different composition. The mixed aqueous surfactant solution Triton X-100, 1.0×10^{-5} M–sodium dodecyl sulfate, 8.1×10^{-4} M–acetone 1:1:5 (*v/v*) was identified as the best mobile phase for specific separation of lysine. This separation can be successfully accomplished in the presence of impurities such as heavy metal cations, inorganic anions, and aliphatic and aromatic amines. The limit of detection of lysine was 0.5 µg per zone. The method has been used for successful identification of lysine in Astymin (Forte) and Alamine (Forte) capsules.

1 Introduction

The urgent need for rapid and selective methods for separation and identification of amino acids has promoted the development of a variety of chromatographic systems for this purpose. The high efficiency, reasonable resolving power, capability of simultaneous analysis of a large number of samples, and simple nature of thin-layer chromatography (TLC) has resulted in its widespread use for analysis of closely related amino acids.

Numerous TLC systems effective in separating amino acids from each other have been well documented in the literature [1–10]. Most literature reports of TLC methods describe use of traditional aqueous, non-aqueous, and mixed-aqueous organic solvents as mobile phases. The use of organic solvents such as benzene, pyridine, carbon tetrachloride, chloroform, and methanol, etc., has been a shortcoming of TLC. As an alternative to complex organic mobile-phase systems, in 1979 *Armstrong* and *Terrill* introduced micellar mobile phases containing aqueous solutions of surfactants for separation of pesticides, decachlorobiphenyl, and nucleosides [11].

Since then, the importance of surfactant-containing mobile phases in chromatographic separations [12–22] has gained impetus. Organic modifiers (e.g. short-chain alcohols) were subsequently added to micellar mobile phases to improve separation selectivity [23, 24].

According to a literature survey, much work has been reported on the use of aqueous solutions containing single surfactants, with or without added organic modifiers, as mobile phases in TLC analysis of inorganic and organic substances [25–30]. Recently the usefulness of mixed surfactant systems containing ionic–nonionic or mixed-ionic micelles has been realized by physical chemists [31–36]. Synergism is often observed in the physicochemical properties of mixed surfactants and sometimes the performance of mixtures is superior to that of the pure surfactant components. Taking into account the excellent performance of aqueous solutions of mixed surfactants, this study was undertaken to examine the resolving power of mixed aqueous surfactants with added acetone in the analysis of amino acids. As a result, we have identified a new TLC system comprising silica gel as stationary phase and 1.0×10^{-5} M Triton X-100 (*t*-octylphenoxypolyethoxyethanol)–8.1×10^{-4} M SDS (sodium dodecyl sulfate)–acetone 1:1:5 (*v/v*) as mobile phase for specific separation of lysine from other essential amino acids.

2 Experimental

All experiments were performed at 30±2°C.

2.1 Chemicals and Reagents

Sodium dodecyl sulfate (SDS) was from BDH India. Triton X-100 (*iso*-octylphenoxy polyethoxyethanol), abbreviated TX-100 in this paper, and the essential amino acids L-lysine (L-lys), L-valine (L-val), L-isoleucine (L-Ile), DL-threonine (DL-Thr), L-methionine (L-met), L-leucine (L-leu), DL-phenylalanine (DL-phe), and DL-tryptophan (DL-Trp) were from CDH, India. Acetone and butan-2-one were from Qualigens, India. Silica gel G (batch no. MK6M562890) and ninhydrin were from Merck, India.

Test solutions of the amino acids (1%) were prepared in demineralized doubled distilled water (DMW). Solutions (1%) of

A. Mohammad and S. Laeeq, Department of Applied Chemistry, Faculty of Engineering and Technology, Aligarh Muslim University, Aligarh, 202002, India.
E-mail: mohammadali4u@rediffmail.com

Table 1

The mobile phases investigated.

Symbol	Composition
M_1	Demineralized double-distilled water (DMW)
M_2	Acetone
M_3	DMW + acetone, 1:1 (*v/v*)
M_4	DMW + acetone, 1:5 (*v/v*)
M_5	1.0×10^{-5} M TX-100
M_6	8.1×10^{-4} M SDS
M_7	1.0×10^{-5} M TX-100 M + acetone, 1:5 (*v/v*)
M_8	8.1×10^{-4} M SDS + acetone, 1:5 (*v/v*)
M_9	1.0×10^{-5} M TX-100 + 8.1×10^{-4} M SDS, 1:1 (*v/v*)
M_{10}	1.0×10^{-5} M TX-100 + 8.1×10^{-4} M SDS, 1:1 (*v/v*)
M_{11}	1.0×10^{-5} M TX-100 + 8.1×10^{-4} M SDS + acetone, 1:1:1 (*v/v*)
M_{12}	1.0×10^{-5} M TX-100 + 8.1×10^{-4} M SDS + acetone, 1:1:2 (*v/v*)
M_{13}	1.0×10^{-5} M TX-100 + 8.1×10^{-4} M SDS + acetone, 1:1:3 (*v/v*)
M_{14}	1.0×10^{-5} M TX-100 + 8.1×10^{-4} M SDS + acetone, 1:1:5 (*v/v*)
M_{15}	1.0×10^{-5} M TX-100 + 8.1×10^{-4} M SDS + acetone, 2:1:1 (*v/v*)
M_{16}	1.0×10^{-5} M TX-100 + 8.1×10^{-4} M SDS + acetone, 3:1:1 (*v/v*)
M_{17}	1.0×10^{-5} M TX-100 + 8.1×10^{-4} M SDS + butan-2-one, 1:1:5 (*v/v*)

the cations Hg^{2+}, Pb^{2+}, Cu^{2+}, Th^{4+}, Zn^{2+}, Fe^{3+}, Cd^{2+}, Cr^{6+}, Ni^{2+}, Co^{2+}, Mo^{6+}, Al^{3+}, and Tl^{3+} were prepared from the metal chlorides, nitrates, or sulfates and contained a small amount of the corresponding acid to prevent hydrolysis. Aqueous solutions (1%) of the anions Cl^-, SO_4^{2-}, NO_3^-, CN^-, PO_4^{3-}, I^-, and ClO_4^- were prepared from the sodium or potassium salts. Solutions (1%) of the amines naphthylamine, methylamine, diphenylamine, tri-*n*-butylamine, *tert*-butylamine, and *m*-phenylenediamine were prepared in methanol. Drug samples were dissolved in 10 mL DMW.

Aqueous solutions of TX-100 and SDS were prepared in DMW. The concentrations of the surfactants were kept below their critical micelle concentrations (CMC; 1.0×10^{-4} M for TX-100 and 8.1×10^{-3} M for SDS). Ninhydrin solution (0.3%) in acetone was used to detect all the amino acids.

2.2 Chromatography

Silica gel G was mixed with DMW in the ratio 1:3 and shaken until a homogenous slurry was obtained. This slurry was immediately coated as 0.25 mm layers on 20 cm × 3.5 cm glass plates by use of a Toshniwal (India) TLC applicator. The plates were dried at room temperature then activated by heating at 100 ± 2°C for 1 h in an electrically controlled oven. After activation the plates were cooled to room temperature then stored in a closed chamber at 30°C before use.

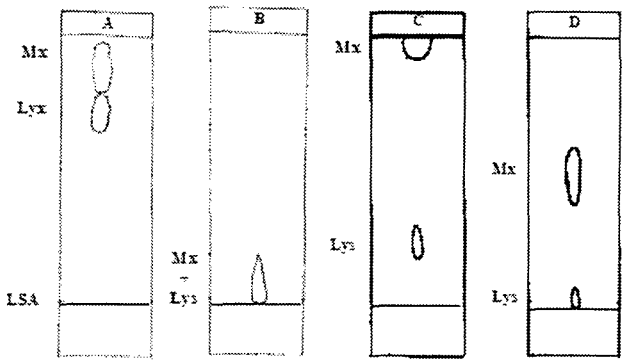

Figure 1

Chromatograms showing the separation of lysine from other amino acids on silica gel layers developed with aqueous mobile phase systems containing acetone: A, DMW; B, acetone; C, DMW–acetone 1:1 (*v/v*); D, DMW–acetone 1:5 (*v/v*). Mx, mixture of seven amino acids; Lys, L-lysine; LSA, line of sample application.

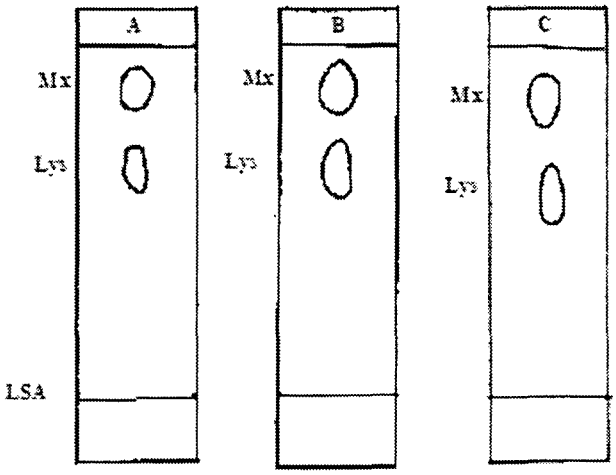

Figure 2

Chromatograms showing the separation of lysine from other amino acids on silica gel layers developed with aqueous solutions of single and mixed surfactants as mobile phases: A, 1.0×10^{-5} M TX-100; B, 8.1×10^{-4} M SDS; C, 1.0×10^{-5} M TX-100 plus 8.1×10^{-4} M SDS, 1:1 (*v/v*); other abbreviations as for Figure 1.

Test solutions (0.2 µL) were applied to the plates, approximately 2 cm above the lower edge, by means of micropipets (Tripette 0783178; Germany). The spots were dried at room temperature (30°C) then chromatography was performed in 24 cm × 6 cm glass jars with lids. The mobile phases investigated are listed in **Table 1**. Chambers were saturated with mobile phase vapor for 10 min before introduction of the plates. Plates were developed by the ascending technique; the development distance was always 10 cm. After development the plates were withdrawn from the jars and dried at room temperature. A glass sprayer was then used to apply ninhydrin and the plates were then heated at 70°C to locate the positions of the analyte spots. R_L (R_F of the leading edge) and R_T (R_F of the trailing edge) values were determined for detected spots and R_F values for amino acids were calculated by use of the formula $R_F = 0.5(R_L + R_T)$.

For selective separation, equal volumes (1 mL) of all the essential amino acid solutions were mixed and 0.2 µL of the resulting mixture was applied to an activated TLC plate. The plate was developed with mobile phase M_{14}, the spots were detected, and

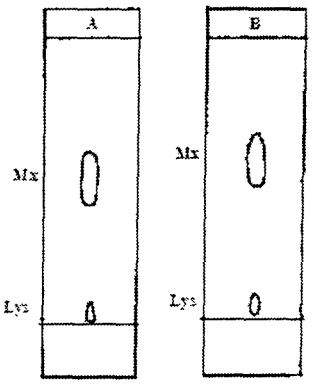

Figure 3

Chromatograms showing the separation of lysine from other amino acids on silica gel layers developed with aqueous solutions of a single surfactant containing acetone as mobile phase: A, 8.1 × 10^{-4} M SDS plus acetone, 1:5 (*v/v*); B, 1.0 × 10^{-5} M TX-100 plus acetone, 1:5 (*v/v*); other abbreviations as for Figure 1.

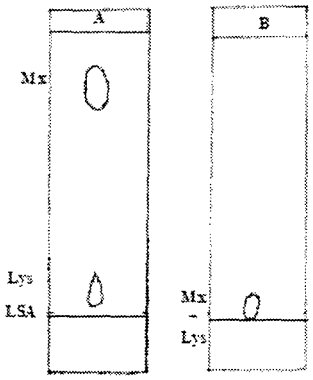

Figure 5

Comparison of chromatograms showing the separation efficiency of mobile phases containing acetone and butan-2-one on silica gel layers: A, 1.0 × 10^{-5} M TX-100 plus 8.1 × 10^{-4} M SDS plus acetone, 1:1:5 (*v/v*); B, 1.0 × 10^{-5} M TX-100 plus 8.1 × 10^{-4} M SDS plus butan-2-one, 1:1:5 (*v/v*); other abbreviations as for Figure 1.

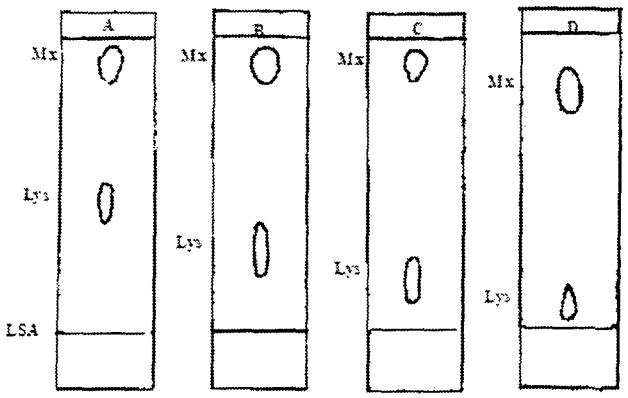

Figure 4

Chromatograms showing the separation of lysine from other amino acids on silica gel layers developed with surfactant-containing mobile phases: A, 1.0 × 10^{-5} M TX-100 plus 8.1 × 10^{-4} M SDS plus acetone, 1:1:1 (*v/v*); B, 1.0 × 10^{-5} M TX-100 plus 8.1 × 10^{-4} M SDS plus acetone, 1:1:2 (*v/v*); C, 1.0 × 10^{-5} M TX-100 plus 8.1 × 10^{-4} M SDS plus acetone, 1:1:3 (*v/v*); D, 1.0 × 10^{-5} M TX-100 plus 8.1 × 10^{-4} M SDS plus acetone, 1:1:5 (*v/v*); other abbreviations as for Figure 1.

the R_F values of the separated lysine and the other amino acids (as a mixture) were calculated.

The limit of detection of lysine was determined by spotting 0.2 µL of its solution on a TLC plate, developing the plate with mobile phase M_{14}, and visualizing the spot with ninhydrin. This process was repeated with successive reduction of the amount of lysine until no spot was detected. The minimum amount of lysine just visible on the TLC plate was taken as the limit of detection.

To examine the effect of the presence of cationic, anionic, and amino compounds (amines) as impurities on the separation of lysine, a solution of the foreign substance (cations, anions, or amine; 0.2 µL) was spotted on the silica layer followed by spotting of the mixture (0.2 µL) of the amino acids. After drying of the spot, TLC was performed with mobile phase M_{14}. Chromatography was performed as described above and the R_F values of lysine and the mixture of other amino acids were calculated after visualization with ninhydrin.

To examine the effect of ageing of mobile phase M_{14} on the separation, the samples were spotted on activated TLC plates, developed with freshly prepared mobile phase (M_{14}), and R_F values were calculated. The same process was repeated at different intervals for 24 h, using the same batch of mobile phase M_{14}, and the R_F values were then calculated and compared.

3 Results and Discussion

The purpose of this investigation was to develop a simple TLC method for specific separation of lysine from other essential amino acids. With this intention all the amino acids (Lys, Val, Ile, Thr, Met, Leu, Phe, and Trp) were chromatographed on silica gel layers using seventeen mobile phases, and optimum experimental conditions were established for identification of lysine in the presence of the other acids in the pharmaceutical formulations Astymin (Forte) and Alamine (Forte) after preliminary separation from other components. The proposed method is important, because lysine is a useful constituent of dietary supplements. On the basis of the results obtained the possibilities of separation of lysine from a mixture with the seven other acids were examined. The separation patterns are shown in the figures. From these results several trends are noticeable.

1. Lysine is barely separated from the other amino acids by use of DMW as mobile phase (**Figure 1A**). The separation is also impossible with pure acetone as mobile phase, because all the amino acids, including lysine, reside at the point of application (Figure 1B). Surprisingly, acetone–water mixtures (1:1 and 5:1, *v/v*) are capable of separating lysine from the other amino acids (Figures 1C and 1D).

2. Aqueous solutions of a single surfactants (1.0 × 10^{-5} M TX-100 or 8.1 × 10^{-4} M SDS) or a mixture of surfactants (1.0 × 10^{-5} M TX-100 plus 8.1 × 10^{-4} M SDS) are not very effective and lead to poor separation of lysine (**Figure 2**).

3. Aqueous surfactant solutions with added acetone (mobile phases M_7–M_{17}) result in better chromatographic performance, increasing the resolution of the two adjacent spots. Compared with single-surfactant–acetone mobile phases (**Figure 3**), mixed-surfactant–acetone mobile phases (**Figure 4**) enable bet-

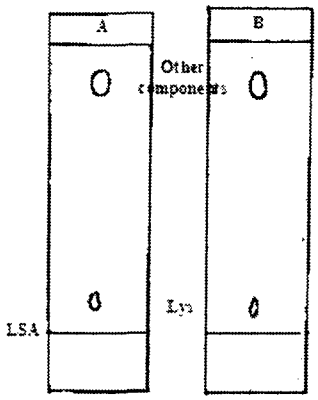

Figure 6

Chromatograms showing the separation of lysine from other components of: A, Astymine (Forte) and B, Alamine (Forte) on silica gel layers developed with 1.0 × 10^{-5} M TX-100 plus 8.1 × 10^{-4} M SDS plus acetone, 1:1:5 (*v/v*); other abbreviations as for Figure 1.

Table 2

Separation of lysine from a mixture of essential amino acids (Mx) in the presence of cations, anions, and amines. The stationary phase was silica gel G and the mobile phase M_{14}.

Impurities	R_F of lysine	R_F of Mx
Cations		
Hg^{2+}, Pb^{2+}, Cu^{2+}, Th^{4+}, Zn^{2+}, Fe^{3+}, Cd^{2+}, Cr^{6+}, Ni^{2+}, Co^{2+}, Mo^{6+}, Al^{3+}, Tl^{3+}	0.08–0.18	0.80–0.90
Anions		
Cl^-, SO_4^{2-}, NO_3^-, CN^-, PO_4^{3-}, I^-, ClO_4^-	0.05–0.16	0.80–0.95
Amines		
Naphthylamine, methylamine, diphenylamine, tri-*n*-butylamine, *tert*-butylamine, and *m*-phenylenediamine	0.05–0.19	0.80–0.95

ter separation. This observation is in agreement with our earlier findings – mixed surfactants were found to enable separation of aromatic amines [37]. The better chromatographic performance of the mixed surfactant solution may be attributed to specific interactions providing an altered microenvironment for separation. It is interesting to note from Figures 4A–4D that the chromatographic performance of aqueous mixed-surfactant systems was found to depend upon the amount of acetone added. It is clear from Figure 4 that increasing the volume of acetone in the mobile phase results in increased resolution. An ion-pair mechanism probably operates during the separation process.

4. The excellent separation of lysine achieved in the presence of acetone (**Figure 5A**) could not be achieved with aqueous mixed surfactant solutions containing butan-2-one (Figure 5B).

The results presented in **Table 2** clearly indicate that cations, anions, and amines do not affect the separation of lysine and it was always possible to isolate it from the other acids.

It was also observed that mobile phase, M_{14} containing mixed surfactants with added acetone can be used continuously for 24 h without any change in the R_F of lysine separated from its mixture with other amino acids. This shows the mobile phase is stable, without significant interactions among its components. The smallest possible amount of lysine visually detectable was 0.5 μg per zone on silica TLC plates developed with M_{14}.

This study clearly reveals the better chromatographic performance of surfactant-mediated mobile phases in the presence of organic modifiers (acetone). This observation is in agreement with those from our previous study [13], in which acetone was identified as the most effective additive at 10% concentration with 3% Brij for separation of heavy metal cations on cellulose layers. Organic modifiers have been found to improve separation efficiency by reducing adsorption of the surfactant on the stationary phase and altering the polarity of the mobile phase.

3.1 Application

The practical utility of this method was examined by analysis of two commercially available pharmaceutical products Astymin and Alamine (both from (Forte). It was concluded the method is suitable for identification of lysine in pharmaceutical formulations after preliminary separation from the other components (**Figure 6**).

4 Conclusion

A new mobile phase system containing mixed aqueous surfactants Triton X-100 and sodium dodecyl sulfate and acetone has been established for specific separation of lysine on silica gel layers. The proposed method can be used to identify the presence of lysine in pharmaceutical capsules.

Acknowledgment

The authors are grateful to The Chairman, Department of Applied Chemistry, Faculty of Engineering and Technology, Aligarh Muslim University, Aligarh, India, for providing research facilities.

References

[1] *A. Mohammad. S. Hena*, and *S.A. Bhawani*, Indian J. Chem. **45** (2006) 1663–1666.

[2] *S. Khawas* and *S. Laskar*, J. Planar Chromatogr. **16** (2003) 165–166.

[3] *A. Mohammad* and *S. Hena*, Chromatography (Japan) **25** (2004) 111–118.

[4] *S.A. Nabi* and *M.A. Khan*, Acta Chromatogr. **13** (2003) 161–171.

[5] *A.J. Mota, E. Castellanos*, and *E. Juaristi*, Org. Prep. Proc. Int. **35** (2003) 414–417.

[6] *S. Khawas* and *S. Laskar*, J. Planar Chromatogr. **16** (2003) 163–166.

[7] *A. Patrut, C. Marutoiu, O.F. Marutoiu*, and *M.I. Moise*, J. Planar Chromatogr. **13** (2002) 151–153.

[8] *A. Mohammad* and *V. Agrawal*, Indian J. Chem. **40** (2001) 1130–1134.

[9] *L. Lepri, V. Coas*, and *P.G. Desideri*, J. Planar Chromatogr. **5** (1992) 175–178.

[10] *R. Bhushan* and *J. Martins*, Amino Acids and Their Derivatives. In: *J. Sherma* and *B. Fried* (Eds) Handbook of Thin Layer Chromatography, 3rd edn, Marcel Dekker, 2003, pp. 373–415.

[11] *D.W. Armstrong* and *R.Q. Terrill*, Anal. Chem. **51** (1979) 2160–2163.

[12] *M.G. Khaledi*, Trends Anal. Chem. **7** (1988) 293–300.

[13] *A. Mohammad, E. Iraqi*, and *I.A. Khan*, J. Surfact. Deterg. **2** (1999) 523–529.

[14] *T. Okada*, J. Chromatogr. A **780** (1997) 343–360.

[15] *G.M. Janini* and *H.J. Issaq*, J. Liq. Chromatogr. Related Technol. **15** (1992) 927–960.

[16] *M.F. Borgerding, R.L. Williams, W.L. Hinze*, and *F.H. Quina*, J. Liq. Chromatogr. **12** (1989) 1367–1406.

[17] *Y. Moroi, H. Akisada, M. Saito*, and *R. Matvura*, J. Colloid Interface Sci. **61** (1977) 233–238.

[18] *K. Saitoh*, Kagaku **45** (1990) 884–885.

[19] *M. Almgre* and *S. Swarup*, J. Colloid Interface Sci. **91** (1983) 256–266.

[20] *A.S. Kord* and *M.G. Khaledi*, Anal. Chem. **64** (1992) 1901–1907.

[21] *A. Berthod, I. Girad*, and *C. Gonnet*, Anal. Chem. **58** (1986) 1359–1362.

[22] *A. Berthod, X. Jun, A. Serge*, and *C. Gonnet-Colette*, Can. J. Chem. **74** (1996) 227–286.

[23] *M.M. Bushey* and *J.W. Jorgenson*, Anal. Chem. **61** (1989) 491–493.

[24] *J.G. Dorsey, M.T. De Echegarary*, and *J.S. Landy*, Anal. Chem. **55** (1983) 924–928.

[25] *S.N. Shtykov* and *E.G. Sumina*, J. Anal. Chem. (Russian) **53** (1998) 446–450.

[26] *A. Mohammad, S. Anwar*, and *V. Agrawal*, J. Indian Chem. Soc. **76** (1999) 452–455.

[27] *S.N. Shtykov, E.G. Sumina*, and *N.V.C. Tyurina*, J. Planar Chromatogr. **13** (2000) 266–270.

[28] *A. Mohammad* and *V. Agrawal*, J. Planar Chromatogr. **13** (2000) 210–216.

[29] *M.A. Hernandez-Torres, J.S. Landy*, and *J. G. Dorsey*, Anal. Chem. **58** (1986) 744–747.

[30] *A. Mohammad* and *N. Jabeen*, Indian J. Chem. Technol. **10** (2003) 79 86.

[31] *K.K. Gosh* and *A. Pandey*, J. Indian Chem. Soc. **76** (1999) 191–194.

[32] *A. Shiloach* and *D. Blankschtein*, Langmuir **14** (1998) 1618–1636.

[33] *S. De, V.K. Aswal, P.S. Goyal*, and *S. Bhattacharya*, J. Phys. Chem. B **101** (1997) 5639–5645.

[34] *M.N. Khan*, Micellar Catalysis, Taylor and Francis, Boca Raton, FL, 2007.

[35] *S. Morisada, H. Shinto*, and *K. Higgashitani*, J. Phys. Chem. B **109** (2005) 11762–11769.

[36] *P.M. Claesson, M. Kjellin, O.J. Rojas*, and *C. Stubenrauch*, J. Phys. Chem. **8** (2006) 5501–5514.

[37] *A. Mohammad* and *I.A. Khan*, Indian J. Chem. Technol. **8** (2001) 244–251.

Ms received: August 25, 2007
Accepted: November 7, 2007

Journal of Planar Chromatography **is published in yearly volumes of six issues by**

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ISSN 0975-413X

Application of Water-in-Oil Microemulsion for Chromatographic Study of Different Groups of Organic Compounds

Ali Mohammad*, Sameen Laeeq

Chromatography Research Laboratory, Department of Applied Chemistry, Faculty of Engineering and Technology, Aligarh Muslim University, Aligarh, U.P., India

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Abstract

Thin-layer chromatographic procedures are described for the analysis and separation of a variety of organic molecules (amino acids, B-complex vitamins, purines and pyrimidines, neutral lipids, polar sugars and dyes) using water-in-oil-microemulsion containing N-cetyl-N, N, N trimethyl ammonium bromide (CTAB) as mobile phase. High performance thin layer chromatographic plates of silica gel G and cellulose were used as stationary phases. The proposed method is suitable for selective separation of bromocresole green from other dyes on cellulose HPTLC plates. Furthermore, vitamins (thiamine, pyridoxine and riboflavin in marketed formulations) were successfully resolved on silica HPTLC plates. Effect of other biomolecules as impurities was investigated on simultaneous separation of thiamine, pyridoxine riboflavin from their mixtures. The well intense colored compact spots for all compounds under study were realized.

Key words: HPTLC, CTAB, microemulsion, biomolecules, separation, vitamins

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Introduction

The growing interest in microemulsion (swollen-micelles) as drug delivery vehicles arises mainly from their physicochemical properties such as transparency, low viscosity, and thermodynamic stability over a range of temperature interval and high solubilization capacity [1]. The possibility of increased solubility of sparingly soluble drugs in microemulsion is of great interest because of the improved therapeutic efficacy of the drug, reduction in the volume of the vehicle and minimization of the toxic side effects [2].

The original application of oil-in-water micro-emulsion in bioanalysis was reported by Berthod et al. when they separated a series of alkylbenzene and screened 11 drugs illegally used in sports [3]. A number of publications on the potential use of microemulsion (ME) as a separation media in HPLC [4], micellar liquid chromatography [5], capillary electrophoresis [6] and microemulsion electrokinetic chromatography (MEKC) have appeared recently [7]. In addition, other application of ME is tertiary oil recovery, cosmetic, lubrication; enzyme catalysis, chemical synthesis etc. were also reported in literature [8, 9].

MEs contain nanometer-sized surfactant coated droplets of oil suspended in water, referred to as oil-in-water microemulsion and vice-versa for water-in-oil microemulsion. In ME liquid chromatography, the surfactant is added in the excess of the critical micelle concentration (CMC) which resulted in large amount of micelles in the mobile phase in which the analyte partitions within the micelles rather than adsorbs onto the stationary phase.

The versatile nature of microemulsion generated renewed interest of chemists to utilize it in chromatographic procedures [10]. Literature suggests that MEs were used as mobile phase to investigate the retention behavior of nitroanilines, amino acids, herbal plant extracts, antibiotics etc. [11-14].

These micellar media seemed to offer another viable clean alternative to traditional organic solvents. Previous studies on thin layer chromatography (TLC) of sugar, amino acids, vitamins, lipids, dyes, purines and pyrimidines utilized hydro-organic, mixed-organic and aqueous surfactants with organic additives as mobile phases for their separation and identification.

So, far no work has been reported for the identification of common sugars by HTLC using water-in-oil ME as eluent. Furthermore, the present study also provides the general applicability of water-in-oil microemulsion in HTLC analysis of compounds belonging to different groups such as vitamins, amino acids, purines and pyrimidines, lipids and dyes.

Results and Discussion

Chromatography of six groups of organic compounds (amino acids, vitamins, sugars, lipids, dyes, purines and pyrimidines) was performed with water-in-oil microemulsion containing CTAB as eluent on two different stationary phases i.e. silica gel G and cellulose HPTLC plates. The following trends were noticed.

(a) All amino acids were strongly retained on both silica and cellulose plates and thus resolution of amino acids with microemulsion as mobile phase is not possible.
(b) All polar sugars, co-migrate without any possible separation on silica gel HPTLC plates showing low mobility ($R_F = 0.29$).
(c) Alike amino acids, polar purines and pyrimidines show high affinity towards both the adsorbents (silica gel and cellulose) and cannot be resolved from their mixtures.
(d) All lipids being hydrophobic well moved with elution window on silica gel G or cellulose showing significant mobility ($R_F = 0.98$) and thus imposing the restriction on their mutual separation. However, they can be easily separated from other organic compounds (amino

acids, vitamins, sugars, dyes, purines and pyrimidines) having lower R_F value.

(e) A fair separation of B-complex vitamins (thiamine hydrochloride, riboflavin and pyridoxine hydrochloride), is always possible on silica HPTLC plate with water-in-oil ME system. For these vitamins, the order of R_F values, given in parenthesis, was thiamine (0.14) < pyridoxine (0.27) < riboflavin (0.44).

The effect of impurity of other bioorganic molecules (lipids, amino acids, sugars and purines & pyrimidines) on the separation of vitamins was also examined. In the presence of lipids, sugars purines and pyrimidines, the mutual separation is always possible but uracil is the exception which hampers the separation. Amongst amino acids, glutamic acid and tyrosine hurt the mutual separation by producing a singly tailed spot near the point of application as a result of co-migration of these vitamins (thiamine, pyridoxine, riboflavin) in the sample. However, in the presence of other amino acids, all the three vitamins are well resolved on single TLC plate. Thus, for vitamins silica gel (inorganic adsorbent) is more efficient stationary phase compared to cellulose (organic adsorbent) because on PEI cellulose HPTLC layers, the separation of these three vitamins could not be achieved. The developed method is applicable to identification of B-complex vitamins in marketed formulation as evident from results presented in Table 1.

Table 1: Identification of vitamins on silica HPTLC plates using water-in-oil micro - emulsion containing CTAB.

	R_F Value		
Sample	Thiamine	Riboflavin	Pyridoxine
B_1	0.08	0.27	0.40
B_2	0.08	0.27	0.41

(f) All dyes were found to stay near the point of application on silica HPTLC plates and hence they cannot be separated. However, a selective separation of bromocresol green (R_F = 0.89) is possible from all cationic (malachite green, methylene blue, brilliant green, rhodamine B and crystal violet) and anionic (xylenol orange, bromocresol green, alizarin red S, congo red, pyrocatechol violet) dyes ($R_F \approx 0.01$) on cellulose HPTLC plate.

Almost all the analytes produce well compact spots near or at the point of application on silica HPTLC, which shows a strong affinity of polar analytes with polar silica gel. Low mobility of polar analytes may be attributed to their strong attraction to negative silanol groups on the silica gel surface [15].

Materials and methods

Experimental

Chemicals and materials

Silica gel 60 F_{254} HPTLC plates (1.05715.001) and PEI Cellulose F_{254} (1.05579.0001) were procured from Merck, Germany. n-Pentanol (Acros organics, New Jersey, USA), heptane and

CTAB were of CDH, India. Amino acids (D- glutamic acid, L–serine, D–alanine, L–tyrosine, L–histidine, L–lysine, L–arginine and phenylalanine), sugars (dextrose, D-fructose, D-lactose, D-galactose, D-ribose and D-xylose), lipids (lauric acid, palmitic acid, stearic acid, n-butyric acid and oelic acid), vitamins (thiamine, riboflavin and pyridoxine) and dyes (xylenol orange, bromocresol green, alizarin red S, congo red, pyrocatechol violet, malachite green, methylene blue, brilliant green, rhodamine B and crystal violet) were from CDH, India. Purines (alanine and guanine) and pyrimidines (cytosine, thymine and uracil) were obtained from Himeda (Mumbai), India. All chemicals were of analytical grade. All experiments were performed at 25±2°C.

Preparation of test solutions

1% (w/v) of amino acids, sugars and B-complex vitamins were prepared in double distilled water. In case of pyridoxine or riboflavin few drops of NaOH were added to make clear solution. Purines and pyrimidines (1% w/v) were prepared in water-methanol mixture (60:40, v/v) and few drops of ammonia were added when necessary. Lipids (1% w/v or v/v) in chloroform : ethanol (1:1) were prepared. Dyes (0.1%) were prepared in mixture of double distilled water plus ethanol in 1:1 ratio by volume.

Marketed formulations

Becosules* capsules (B-complex Forte with vitamin C), Pfizer and Becozinc syrup, Dr. Reddy's, Hyderabad, India, analysed by the proposed method.

1.2.Preparation of formulation solutions

Becosules *capsule was finely powdered and dissolved in 10 ml of double distilled water (B_1 sample) and 1 % solution of Becozinc syrup was prepared in double distilled water(B_2 sample).

Detection reagent

Ethanolic solution (0.3%) of ninhydrin was sprayed on the HPTLC plates to locate the position of amino acids. Purple spots appeared on heating the HPTLC plates at 60°C for few minutes.
Sugars were detected by spraying ethanolic orcinol solution on silica HPTLC plates and heating at 110°C for 5-10 minutes. The detected spots appeared as brown- purple for all disaccharides and hexoses except D-fructose (appeared as orange) and blue for pentoses.

All vitamins, purines, pyrimidines and lipids were UV active and can be easily visualized under short UV lamp. Dyes were detected visually according to their original color.

Preparation of mobile phase (Microemulsion)

Water-in-oil ME used as mobile phase was prepared at 25°C by titrating a coarse emulsion of heptane (160 mL), water (8 mL) and CTAB (8 gm) with n-pentanol (25 mL). The microemulsion system was transparent optically clear and remained stable at 25±2°C for several weeks. This was used as mobile phase for six groups of organic compounds.

HPTLC method

Test solutions (1µL) of all analytes were applied on (6 x 6 cm) silica gel 60 F_{254} and PEI cellulose high-performance thin-layer plates with the help of micropipette at about 1 cm above

the lower edge of the plates. The solvent ascent was fixed to 5 cm in all cases for the determination of R_F value of individual analyte. Linear ascending development was carried out in vapor equilibrated Camag TLC twin-trough chamber. The optimized chamber saturation time for the mobile phase was 10 min at room temperature (25±2°C). Subsequent to the development, TLC plates were dried at room temperature. The plates were detected by using appropriate detector for desired analyte. The R_L (R_F of leading front) and R_T (R_F of trailing front) values of each spot were determined and the R_F value was calculated

For the separation of vitamins or dyes from their mixtures, equal volumes of vitamins or dyes were mixed and 1 µL of the resultant mixture was applied on TLC silica gel or cellulose plate. The plate was developed with mobile phase, the spots were detected and the R_F values of the separated spots of vitamins and dyes were calculated. Similarly dyes were also separated on cellulose plates.

For investigating the interference of other bioorganic molecules (amino acid, sugars, lipids, purines and pyrimidines) on the mobility and mutual separation of vitamins, aqueous solutions (as prepared earlier) were used for interference studies. The effect of biomolecules as impurities on the resolution of vitamins from their mixture, 1 µL of the standard test mixture of vitamins was spotted on the plate followed by the spotting of 1 µL of biomolecules considered as impurities. The plates were developed with microemulsion containing CTAB mobile phase, spots were detected under UV lamp and R_F values of the separated vitamins were calculated.

Conclusion

Oil-in-water microemulsion proved to be useful surfactant-mediated system for ternary separation of vitamins (thiamine, riboflavin and pyridoxine) in pharmaceutical preparations. On cellulose layers, bromocresol green was selectively separated from other anionic as well as cationic dyes.

Acknowledgment

The authors are grateful to the Chairman, Department of Applied Chemistry, Aligarh Muslim University, Aligarh, India and University Grant Commission (UGC), New Delhi, India, for providing research facilities and financial assistance respectively.

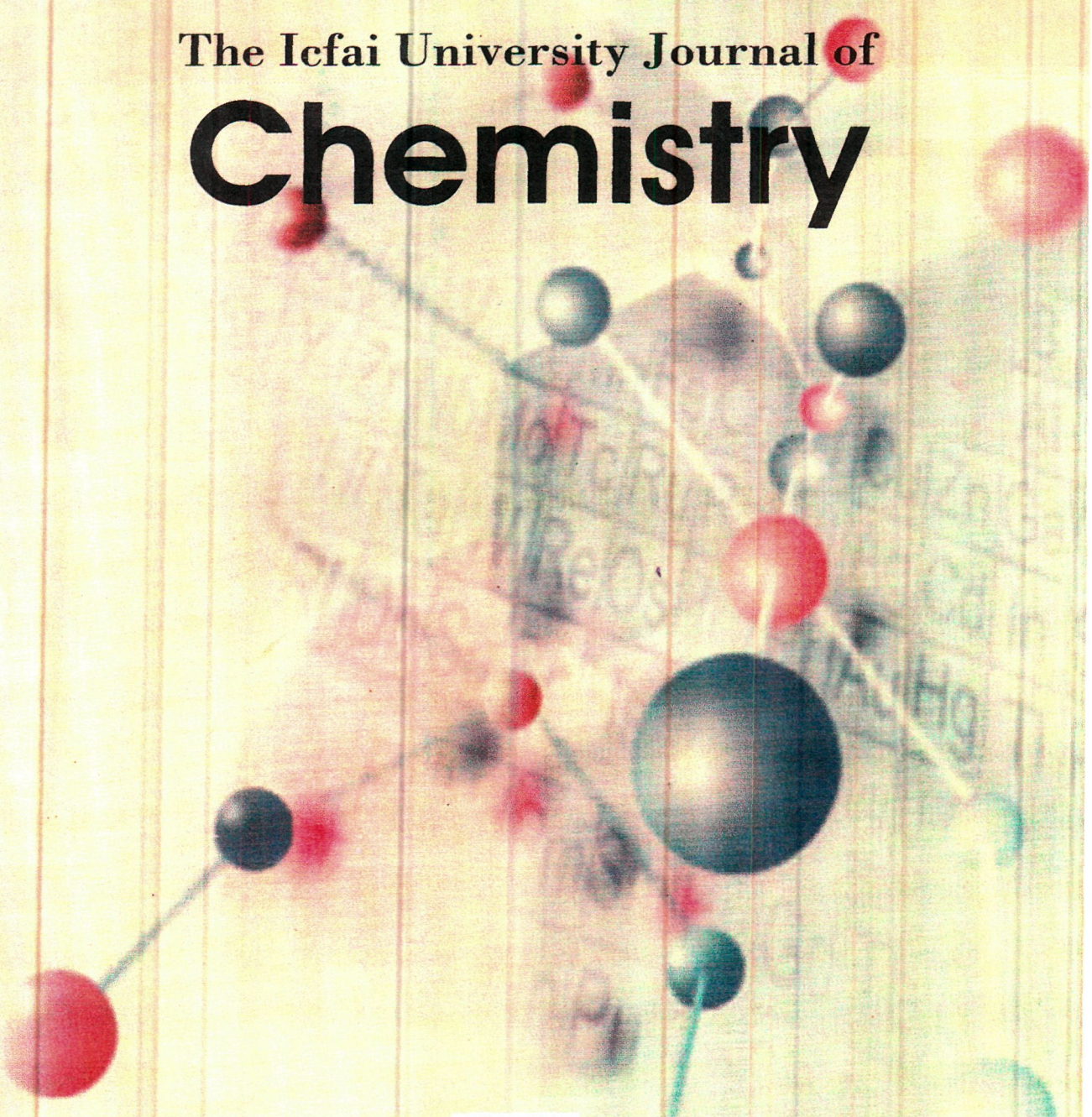
References

[1] P.K. Ghosh, R.S.R. Murthy, *Drug. Del. C.*, **2006,** 3, 167.
[2] R. Ryan, S. Donegan, J. Power, E. McEvoy, K. Altria, *Electrophoresis,* **2009,** 30, 65.
[3] A. Berthod, J.J. Lasesna, I. Carretero, *J. Liq. Chromatogr. Relat. Technol.*, **1992,** 15, 3115.
[4] K. Altria, M. Broderick, S. Donegan, J. Power, *Chromatographia*, **2005,** 62, 341.
[5] M.B. Simon, D.A. Kevin, *J. Sep. Sci.*, **2004,** 27, 1498.
[6] A. Marsh, M. Broderick, K. Altria, J. Power, S. Donegan, B. Clark, *Methods Mol. Biol.*, **2008,** 384, 205.
[7] M. Murillo-Arbizu, E. Gonzatez-Penas, S.H. Hansen, S. Amezqueta, J. Ostergaard, *Food*

Chem. Toxicol., **2008,** 46, 2251.
[8] B.K. Paul, S.P. Moulik, *Curr. Sci.*, **2001,** 80, 990.
[9] K.D. Altria, *J. Chromatogr. A*, **2000,** 892, 171.
[10] G.M. Eccleston, Emulsion and Microemulsions, (IInd Ed., J. Swarbrick, J.C. Boylan, Marcel Dekker, Inc., New York, 2002) 1080.
[11] A. Mohammad, S. Hina, *Acta Chromatographica*, **2005,** 15, 238.
[12] A. Mohammad, V. Agrawal, S. Kumar, *J. Planar Chromatogr.*. **2003,** 16, 220.
[13] S.F. Cui, B.Q. Fu, F.S.C. Lee, X.R. Wang, *J. chromatogr. B,* **2005,** 828, 33.
[14] A. Malenovic, D. Ivanovic, M. Medenica, B. Jancic, S. Markovic, *J Sep. Sci.* **2004,** 13, 1087.
[15] B.A. Olsen, *J. Chromatogr. A*, **2001,** 913, 113.

Vol. I No. 2 December 2008

The Icfai University Journal of Chemistry



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Identification and Simultaneous Separation of Six Hydrophilic Therapeutic Vitamins by Micellar Thin Layer Chromatography

Ali Mohammad* and Sameen Laeeq**

A novel micellar thin layer chromatographic method for simultaneous separation and on-plate identification of B-group vitamins along with ascorbic and folic acids has been developed. A hybrid mobile phase constituting a mixture of 4% aqueous Sodium Dodecyl Sulphate (SDS) + acetonitrile (1:2, v/v) was identified as the most favorable for the resolution of multicomponent mixture of vitamins on silica high performance Thin Layer Chromatography (TLC) plates (silica gel $60F_{254}$ catalog no. 1.05554, Merck, Germany). The resolved spots were identified by visualization under ultraviolet radiation (I = 254) in a closed UV cabinet. The effects of type of sample solvent, concentration of surfactant (SDS), volume ratio of acetonitrile in the mobile phase, and the presence of essential amino acids in the sample have been examined. This method is simple, precise, sensitive and useful for the analysis of vitamins B_1, B_2, B_6, B_{12}, ascorbic and folic acids present in marketed pharmaceutical formulation.

Keywords: Hydrophilic vitamins, Micellar TLC, Anionic surfactant, Vitamin identification

Introduction

Thin Layer Chromatography (TLC) represents one of the most important analytical techniques for identification and simultaneous separation of organic compounds (Wagner and Bladt, 1995; Sherma and Fried, 2003; Cimpoiu and Hosu, 2007; and Mohammad and Laeeq, 2007). Vitamins, the amphipathic molecules, are the precursors of various metabolic pathways in every living organism. These organic molecules essentially play important functions as coenzymes and antioxidants. These occur in almost all dietary products. Their deficiency may lead to numerous disorders. To overcome their loss in pathological conditions, they can be used in the form of various therapeutic multivitamin dosages, which are composed of B-group vitamins, folic acid and ascorbic acids, biotin, calcium pantothenate fortified with

* Senior Faculty Member, Analytical Research Laboratory, Department of Applied Chemistry, Faculty of Engineering & Technology, Aligarh Muslim University, Aligarh 202002, India. E-mail: mohammadali4u@rediffmail.com

** Research Scholar, Analytical Research Laboratory, Department of Applied Chemistry, Faculty of Engineering & Technology, Aligarh Muslim University, Aligarh 202002, India. E-mail: sameen645@rediffmail.com

minerals and antioxidants. For the analysis of six hydrophilic vitamins, this new micellar TLC procedure is designed.

According to literature, various methods like High Performance Liquid Chromatography (HPLC) using diode array detector in commercial multivitamin preparation (Chu K-On and Tin, 1998); HPLC (Huang X *et al., 1999),* Reverse Phase-HPLC (RP-HPCL) (Bhushan and Meenakshi, 2002), TLC using fiber-optic fluorometric quantitation (Navas *et al.*, 1993) and quantitative determination by fluorescence quenching (Srinivas *et al.*, 1992; and Perisic *et al.*, 1995), High-Performance Thin Layer Chromatography (HPTLC) (Kartsova and Koroleva, 2007) and RP-TLC (Baranowska and Kadziolka, 1996) have been used in the analysis of vitamins. Most of thin layer chromatographic procedures developed for their analysis involve the use of organic or mixed aqueous organic mobile phases (Watanabe and Miyamoto, 2006). Since the first report (Armstrong and Terrill, 1979) on the use of aqueous solution surfactant as mobile phase, several workers (Dorsey *et al.*, 1983) have utilized it in the analysis of closely related organic as well as inorganic substances by ion-pair and micellar TLC (Kord and Khaledi, 1992; Mohammad and Jabeen, 2003; and Mohammad *et al.*, 2004).

The excellent chromatographic performance of surfactant-mediated systems in the presence of small amounts of added organic solvents has generated renewed interest in chromatographers (Khaledi, 1988; Borgerding *et al.*, 1989; and Shtykov *et al.*, 1999). The favorable features of surfactants as modifiers of mobile and stationary phases in TLC have been admirably documented in recent review by Sumina *et al.* (2003) in recent review.

The most interesting feature of surfactants solutions is their dual hydrophobic and hydrophilic nature that provides electrostatic as well as hydrophobic interactions leading to unusual separation possibilities (Hernandez-Torres *et al.*, 1986; and Berthod and Carvalho, 1992). The capability of simultaneous separation of ionic and non-ionic compounds is another fascinating feature of micellar systems.

Encouraged by the above-mentioned advantageous features of micellar solution systems, a new TLC system consisting of silica gel $60F_{254}$ HPTLC plates in combination with aqueous micellar SDS, an anionic surfactant containing acetonitrile as organic modifier has been identified as most favorable for identification and separation of B-group vitamins along with folic and ascorbic acids under UV radiations ($\lambda = 254$ nm). Sodium Dodecyl Sulphate (SDS) was selected because of the fact that amongst the various micelles of surfactants (Almgren and Swarup 1983; and Mohammad and Sirwal, 2003) it was found to be the most efficient, especially with silica gel.

The present study aims to develop a reliable and efficient micellar TLC method for simultaneous separation and detection of B_1, B_2, B_6, B_{12}, ascorbic acid and folic acid. The proposed method has been applied to the analysis of multivitamin formulation (Becosules* capsules). The limit of detection of vitamins is listed in Table 1.

Table 1: Limit of Detection of Vitamins

Vitamin	Limit of Detection (μ g)
B_1	0.200
B_2	0.002
B_6	0.400
B_{12}	0.040
C	0.200
Folic Acid	0.020

Experimental Details

All experiments were performed at 30 ± 2 °C.

Apparatus

HPTLC aluminum sheet of silica gel 60 F_{254} plates (BN 1.05554 from Merck, Germany) and TLC twin-trough chamber (Camag, Muttenz, Switzerland) lined with lid were used.

Cetyltrimethyl Ammonium Bromide (CTAB) was procured from CDH, India. Acetonitrile (Lichrosolv) were from Merck, India. SDS was purchased from BDH, India. All reagents were of analytical grade.

Test Solutions

Standard aqueous test solutions (0.01%) of vitamin B_2, (0.1%) of folic acid, (0.2%) of B_{12}, (1.0%) of ascorbic acid and vitamin B_1 and (2.0%) of vitamin B_6 were prepared in double distilled water.

Sample Preparation

For the sample preparation, standard aqueous test solutions (0.3 mL) of each of the listed vitamins (Table 2) were mixed, and 0.50 mL of this mixture was used for chromatography.

Table 2: Vitamins Used

Vitamin	Common Name	Chemical Name
B_1	Thiamine hydrochloride (CDH, India)	3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl) -4-methyl thiazolium chloride hydrochloride
B_2	Riboflavin (CDH, India)	3, 10-dihydro-7, 8-dimethyl-10-[(2S, 3S, 4R)-2, 3, 4, 5-tetrahydroxypentyl]benzo-[g]pteridine-2, 4-dione;7, 8-dimethyl-10-(1'-D-ribityl)isoalloxazine

(Contd...)

Table 2: Vitamins Used *(...contd)*		
Vitamin	**Common Name**	**Chemical Name**
B_6	Pyridoxine hydrochloride (BDH, India)	hydroxy-6-methyl-3, 4-pyridine dimethanol hydrochloride
B_{12}	Cyanocobalamine (CDH, India)	α-(5, 6-dimethylbenzimidazolyl) cobamidcyanide
C	L-ascorbic acid (CDH, India)	2, 3-endiol-L-gulonic acid-γ-lactone
Folic Acid	Folic acid (CDH, India)	N-[4((2-amino-1, 4-dihydro-oxo-6 pteridinyl)methylamino)benzoyl] -L-glutamic acid

Dosage Formulation

Becosules* capsules (B-complex Forte with vitamin C) Pfizer, India, analyzed by the proposed method has the following composition (mg):

- Thiamine Mononitrate IP 10
- Riboflavin IP 10
- Pyridoxine Hydrochloride IP 3
- Vitamin B12 IP (as Stablets 1:100) 15
- Niacinamide IP 100
- Calcium Pantothenate 50
- Folic Acid IP 1.5
- Biotin USP 100
- Ascorbic acid IP 150

Preparation of Dosage Solutions

Becosules *capsule was finely powdered and dissolved in 10 mL double distilled water (S_1). The resultant solution was spiked with 0.2 mL of cyanocobalamine (0.2%) and 0.2 mL of folic acid (0.1%). This mixture was treated as the commercial analyte sample. For studying the nature of sample solvent, several analyte samples were prepared by dissolving the powder of Becosules* capsule separately in 10 mL of ethanol

(S_1), 0.04 M orthophosphoric acid (S_2), 0.24 M sodium hydroxide (S_3), 0.001 M SDS (S_4) and 0.001 M CTAB (S_5).

Detection

UV radiation (λ = 254 nm) was used to detect all the studied vitamins except B_{12} which was observed visually in sunlight as a pink spot. The plates were photographed under 254 nm using Nikon cool pix 6.2 megapixel digital camera.

Mobile Phases

The solvent systems used as mobile phases are listed in Table 3.

Table 3: List of Mobile Phases Used

Symbol	Composition
M_1	Acetonitrile
M_2	Aqueous SDS solution (4%)
M_3	Aqueous SDS solution 4% + acetonitrile (1:1, v/v)
M_4	Aqueous SDS solution 4% + acetonitrile (1:2, v/v)
M_5	Aqueous SDS solution 4% + acetonitrile (1:3, v/v)
M_6	Aqueous SDS solution 2% + acetonitrile (1:2, v/v)
M_7	Aqueous SDS solution 4% + acetonitrile (2:1, v/v)
M_8	Aqueous SDS solution 6% + acetonitrile (1:2, v/v)
M_9	Aqueous TX-100 solution 4% + acetonitrile (1:2, v/v)
M_{10}	Aqueous CTAB solution 4% + acetonitrile (1:2, v/v)

Procedure

Silica 60 F_{254} HPTLC plates were activated at 60 ± 2 °C in an electrically controlled oven for 20 minutes and stored in closed chamber until used. Spotting of 0.50 µl of sample using micropipette (Tripette 0783178, Germany) was done at 1.0 cm from the base of HPTLC plates. Spots were dried in air and developed in a closed presaturated glass chamber with the desired mobile phase by ascending technique up to the ascent of 5 cm from the point of application. After development, the HPTLC plates were withdrawn from the glass chamber, air dried and then detected under UV light to locate the position of individual vitamin in the form of fluorescing spots.

Results and Discussion

Chromatography of six hydrophilic vitamins was performed using various hybrid mobile phases composed of 4% aqueous SDS and acetonitrile in different volume ratios. The mobility pattern of vitamins, viewed under UV radiations (λ = 254 nm) was found to depend on the composition of the mobile phase. The results obtained during the entire study are summarized in Figures 1-12.

From the results shown in the figures, the following trends are noticeable:

i. With acetonitrile (M_1) as mobile phase, all vitamins remain at the point of application as highly compact and well-formed spots (Figure 1).

ii. With SDS micellar solution (M_2), all vitamins show significant mobility producing diffused spots, which appeared near the solvent front. Cyanocobalamine was the exception, which appeared at the middle of TLC plate. None of the vitamins produces compact spot (Figure 2).

Figure 1: Chromatogram Showing the Mobility Pattern of Vitamins B_{12}, B_1, B_6, B_2, Folic Acid and C from Left to Right. Plate Developed with Mobile Phase M_1

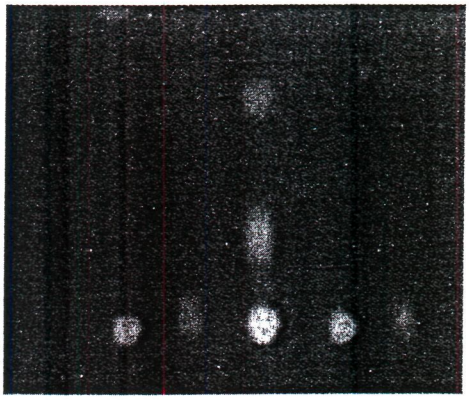
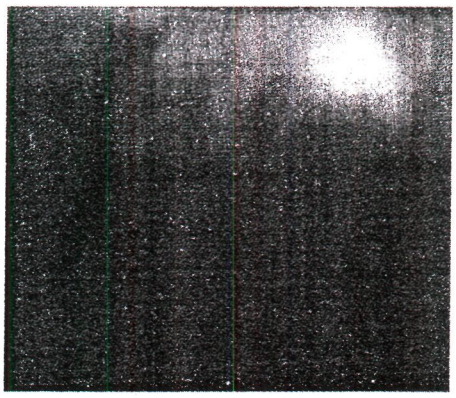


Figure 2: Chromatogram Showing the Mobility Pattern of Vitamins B_{12}, B_1, B_6, B_2, Folic Acid and C from Left to Right Plate Developed with Mobile Phase M_2



iii. As expected, the hybrid mobile phase systems (M_3-M_5) prepared by mixing 4% aqueous solution of SDS and acetonitrile in different volume ratios gave useful results, which can be utilized in chromatographic analysis of vitamins (Figures 3-5). In general, on the increase or decrease of SDS volume in the mobile phases M_6 and M_8, all vitamins tended to smear along the length of the chromatogram rather than move as discrete spot. Out of these mobile

Figure 3: Chromatogram Showing the Mobility Pattern of Vitamins B_{12}, B_1, B_6, B_2, Folic Acid and C from Left to Right. Plate Developed with Mobile Phase M_3

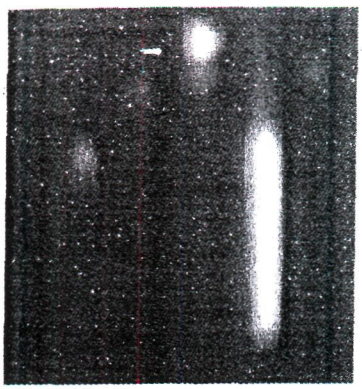


Figure 4: Chromatogram Showing the Mobility Pattern of Vitamins B_{12}, B_1, B_6, B_2, Folic Acid and C from Left to Right. Plate Developed with Mobile Phase M_4

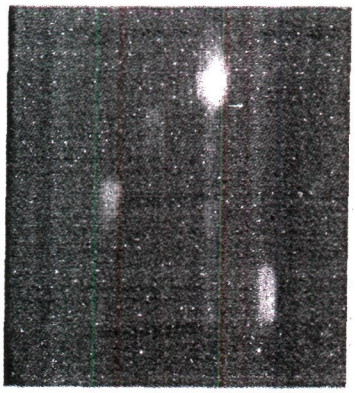


Figure 5: Chromatogram Showing the Mobility Pattern of Vitamins B_{12}, B_1, B_6, B_2, Folic Acid and C from Left to Right. Plate Developed with Mobile Phase M_5

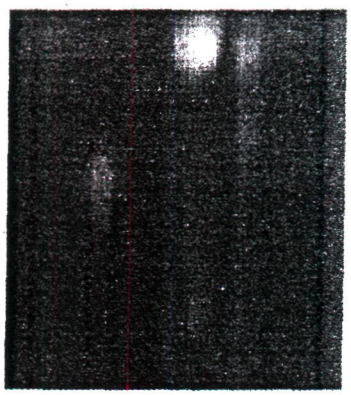
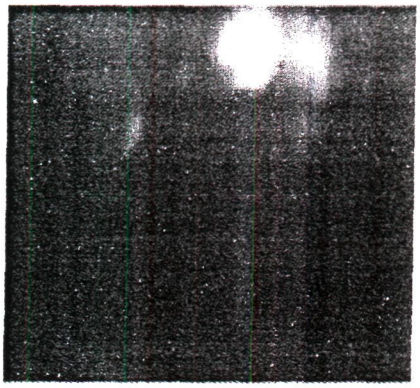


Figure 6: Chromatogram Showing the Mobility Pattern of Vitamins B_{12}, B_1, B_6, B_2, Folic Acid and C from Left to Right. Plate Developed with Mobile Phase M_4



phases, system constituting of 4% aqueous SDS + acetonitrile (1:2, v/v) was found most favorable for the separation and identification of vitamins from their mixture in the sample (Figure 9). The substitution of SDS in M_4 by 4% aqueous TX-100 (a nonionic surfactant) or 4% aqueous CTAB (a cationic surfactant) could not produce useful results (Figures 7 and 8), and hence, the TLC system comprising of HPTLC silica as stationary phase and 4% aqueous SDS plus acetonitrile (1:2, v/v) termed as M_4 in this paper was selected for further study.

Figure 7: Chromatogram Showing the Mobility Pattern of Vitamins B_{12}, B_1, B_6, B_2, Folic Acid and C from Left to Right. Plate Developed with Mobile Phase M_6

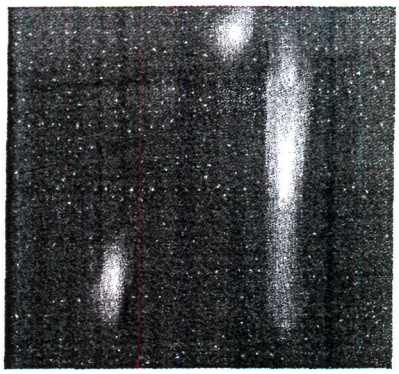
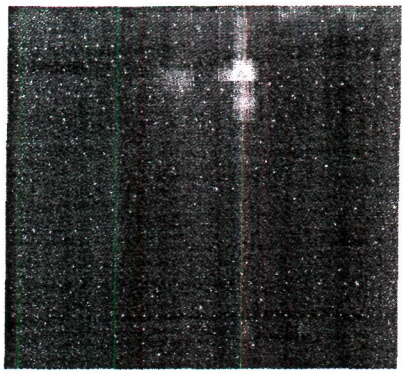


Figure 8: Chromatogram Showing the Mobility Pattern of Vitamins B_{12}, B_1, B_6, B_2, Folic Acid and C from Left to Right. Plate Developed with Mobile Phase M_8



iv. The selected TLC system was tested for its applicability on the marketed formulation (Becosules* capsule) as follows:

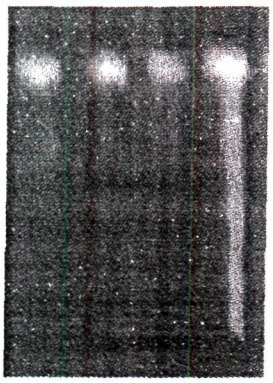
- For identification of vitamins on HPTLC plate, the capsule sample dissolved in double distilled water (S_1) was chromatographed on HPTLC plate and the pattern realized for identification of vitamins is shown in Figure 10. The effect of a few essential amino acids on the separation pattern of vitamins was also examined and the results are presented in Figure 11. The presence of amino acids does not influence the separation and identification of vitamins.

Figure 9: Chromatogram Showing the Separation of Vitamins B_{12}, B_1, B_6, B_2, Folic Acid and C from their Mixture (Standard Sample). Plate Developed with Mobile Phase M_4

Figure 10: Chromatogram Showing the Resolution of Vitamins Present in Becosules Capsule. Plate Developed with Mobile Phase M_4

Figure 11: Effect of Essential Amino Acids as Impurity Added in Becosules Capsule. Plate Developed with Mobile Phase M_4

Figure 12: Effect of Sample Solvents S_2, S_3, S_4 and S_5 from Left to Right for Chromatographic Performance of Becosules Capsule. Plate Developed with Mobile Phase M_4



- In order to understand the effect of sample solvent on chromatographic performance of vitamins, the solution of sample (Becosules *capsule) was prepared in S_2, S_3, S_4 and S_5 separately. The chromatographic behavior of the resultant solution of vitamins is shown in Figure 12. CTAB is the only substance to influence the separation of vitamins. Thus, vitamins can be analyzed in a commercial sample under different solvent conditions. All the samples were found to be stable for a duration of 48 hours as the monitoring of sample at different intervals revealed almost identical chromatograms. It is evident from the figures that the chromatographic patterns of the standard sample solution (Figure 9) and commercial sample solution (Figure 10) are not identical. The difference may be attributed to the presence of other components (biotin, calcium pantothenate and nicotinamide) in the commercial sample.

- The lowest possible detectable amount of vitamins B_1, B_2, B_6 and B_{12}, folic acid and L-ascorbic acid on silica $60F_{254}$ HPTLC plates developed with M_4 ranges from 0.002-0.4 µL. Thus, the proposed method is highly sensitive for on-plate detection of vitamins.

Conclusion

A new micellar mobile phase comprising 4% aqueous SDS + acetonitrile (1:2, v/v) was developed for the rapid analysis of therapeutic vitamins. Being selective, the proposed TLC method could be easily implemented as a reliable analytical tool for the separation and identification of hydrophilic vitamins in pharmaceutical formulations.

Acknowledgment: *The authors thank the Chairman, Department of Applied Chemistry and the University Grants Commission, New Delhi (India) for providing research facilities and financial assistance respectively.*

References

1. Almgren M and Swarup S (1983), "Size of Sodium Dodecyl Sulphate Micelles in the Presence of Additives: Alcohols and Other Polar Compounds", *J. Colloid Interface Sciences*, Vol. 91, No. 1, pp. 256-266.

2. Armstrong D W and Terrill R Q (1979), "Thin Layer Chromatographic Separation of Pesticides, Decachlorobiphenyl and Nucleosides with Micellar Solutions", *Anal. Chem.*, Vol. 51, No. 13, pp. 2160-2163.

3. Baranowska I and Kadziolka A (1996), "RPTLC and Derivative Spectrometry for the Analysis of Selected Vitamins", *Acta. Chromatography*, Vol. 6, pp. 61-71.

4. Berthod A and Carvalho M De (1992), "Oil-in-Water Microemulsions as Mobile Phases in Liquid Chromatography", *Anal. Chem.*, Vol. 6, No. 6, pp. 2267-2272.

5. Bhushan R and Meenakshi A (2002), "Separation of Vitamin B Complex and Folic Acid by HPLC, Normal Phase and Reversed Phase TLC", *National Academy Science Letters (India)*, Vol. 25, Nos. 5 and 6, pp. 159-167.

6. Borgerding M F, Williams R L, Hinze W L and Quina F H (1989), "New Perspectives in Micellar Liquid Chromatography," *J. Liquid Chromatography*, Vol. 12, No. 8, pp. 1367-1406.

7. Chu K-On and Tin K C (1998), "Analysis of Commercial Multi-Vitamin Preparation by HPLC with Diode Array Detector", *Anal. Lett.*, Vol. 31, No. 15, pp. 2707-2715.

8. Cimpoiu C and Hosu A (2007), "Thin Layer Chromatography for the Analysis of Vitamins and Their Derivatives", *Journal Liquid Chromatography & Related Sci.*, Vol. 30, Nos. 5-7, pp. 701-728.

9. Dorsey J G, DeEchegaray M T and Landy J S (1983), "Efficiency Enhancement in Micellar Liquid Chromatography", *Anal. Chem.*, Vol. 55, No. 6, pp. 924-928.

10. Hernandez-Torres M A, Landy J S and Dorsey J G (1986), "Reversed Micellar Mobile Phases for Normal-Phase Chromatography", *Anal. Chem.*, Vol. 58, No. 4, pp. 744-747.

11. Huang X, Gusev I and Horvath C (1999), "Capillary Columns with in Situ Formed Porous Monolithic Packing for Micro High-Performance Liquid Chromatography

and Capillary Electrochromatography", *Journal of Chromatography A*, Vol. 855, pp. 273-290.

12. Kartsova L A and Koroleva O A (2007), "Simultaneous Determination of Water- and Fat-Soluble Vitamins by High Performance Thin Layer Chromatography Using an Aqueous Micellar Mobile Phase", *J. Analytical Chem.*, Vol. 62, No. 3, pp. 255-259.

13. Khaledi M G (1988), "Bioanalytical Capabilities of Micellar Liquid Chromatography" (Review), *Trends Anal. Chem.*, Vol. 7, pp. 293-300.

14. Kord A S and Khalidi M G (1992), "Chromatographic Characteristic of Surfactant-Mediated Separations: Micellar Liquid Chromatography vs. Ion-Pair Chromatography", *Anal. Chem.*, Vol. 64, No. 17, pp. 1901-1907.

15. Mohammad A and Jabeen N (2003), "TLC Studies and Separation of Heavy Metal Cations on Soil Amended Silica Gel Layer Developed with Surfactant-Mediated Solvent Systems", *Indian J. Chem., Technol.*, Vol. 10, No. 1, pp.79-86.

16. Mohammad A and Sirwal Y H (2003), "A New Anionic Micellar Mobile Phase System for the Normal-Phase Thin-Layer Chromatography of Amino Compounds: Simultaneous Separation of Aminophenol Isomers", *Journal of Analytical Chemistry*, Vol. 58, No. 8, pp. 731-739.

17. Mohammad A and Laeeq S (2007), "Mixed Surfactant Enable Separation of Lysine from Other Essential Amino Acids in TLC on Silica Gel", *J. Planar chromatogr.*, Vol. 20, No. 6, pp. 65-69.

18. Mohammad A, Agrawal V and Hena S (2004), "Adsorption Studies of Metal Cations on a Silica Static Flat Bed Using Anionic Micellar Mobile-Phase System Containing Carboxylic Acid :Separation of Coexsisting Iron (III), Copper (II) and Nickel (II) Cations", *Adsorption Science & Technology*, Vol. 22, pp. 89-105.

19. Mohammad A, Iraqi E and Khan I A (1999), "Micellar Thin Layer Chromatographic Separation of Heavy Metal Cations: Effect of Organic and Inorganic Additives on Migration Behaviour", *J. Surfactants Detergr.*, Vol. 2, No. 4, pp. 523-529.

20. Navas D A, Guirado P A and Garcia S F (1993), "Thin-Layer Chromatography and Fiber-Optic Fluorometric Quantitation of Thiamine, Riboflavin and Niacin", *J. Chromatogr.*, Vol. 655, pp. 39-43.

21. Perisic Janjic N U, Poponic M R and Djakovic T J (1995), "Quantitative Determination of Vitamin B Complex Constituents by Fluorescence Quenching After TLC Separation", *Acta. Chromatography*, Vol. 5, pp. 144-150.

22. Sherma J and Fried B (Eds.) (2003), *Handbook of Thin Layer Chromatography*, 3rd Edition, Vol. 89, Marcel Dekkar Inc., New York.

23. Shtykov S N, Sumina E G, Smushkina E V and Tyurina N V (1999), "Normaland Reversed Phase Thin Layer Chromatography of Fluorescein Dyes with Aqueous Micellar Solution as Mobile Phases", *J. Planar chromatogr.*, Vol. 12, No. 2, pp. 129-134.

24. Srinivas B, Srinivasulu K and Vikram (1992), "Separation of Some Water-Soluble Vitamins by Thin Layer Chromatography Using Heulandite as an Adsorbent", *J. Indian Chem. Soc.*, Vol. 69, No. 10, pp. 669-670.

25. Sumina E G, Shtykov S N and Tyurina N V (2003), "Surfactants in Thin-Layer Chromatography", *Journal of Analytical Chemistry*, Vol. 58, No. 8, pp. 720-730.

26. Wagner H and Bladt S (Eds.) (1995), *Plant Drug Analysis: A Thin Layer Chromatography Atlas*, Springer, Berlin, Germany.

27. Watanabe F and Miyamoto E (2006), "TLC of Hydrophilic Vitamins", *Chromatographic Sci. Series (Eng.)*, Vol. 95, pp. 237-250.

Reference # 65J-2008-12-02-01

Vol. I No. 2 December 2008

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Tel: +91(40) 23435-368 to 374; Fax: +91(40) 2335-2521
E-mail: info@iupindia.org; Website: www.iupindia.org